

Timing is everything

If you're a morning person, you know how hard it is to function properly late at night. And don't even think of getting a night owl to talk sense at daybreak. Yet our society largely ignores these important differences.

The early bird catches the worm. *Chi dormi non piglia pesci*. *Morgenstund hat Gold im Mund*. *A quien madruga, Dios le ayuda*. Most Western languages have a proverb that sings the virtues of the 'larks' who are early to rise — or decries the 'night owls' who need a lie-in to recover from their exertions of the night before.

In the pre-electric era, this made sense: people had to make the most of limited daylight. Today, living in an artificially illuminated '24/7' society, there is no good reason to hold earlier risers in especially high esteem. But if we are all to perform optimally, there is every reason to pay more attention to the underlying biological rhythms that determine our daily, or circadian, patterns of activity. Too many of us find ourselves working against our body's natural rhythms, rather than with them. And that's bad news, for both our quality of life and our economic productivity.

Circadian biologists have long known that our daily cycles of sleep and wakefulness, hormone levels and body temperature, vary dramatically in phase between individuals. The norm may be to take eight hours' sleep between 11 p.m. and 7 a.m., but people's natural preferences vary, giving a normal distribution across the population that can put two individuals' preferred bed-times out of synch by up to eight hours. Researchers even have a specific label to describe the phase of an individual's body rhythms: their 'chronotype'.

The phase of our circadian rhythms varies with age, and it is now emerging that it can be seriously disrupted by a variety of diseases

(see page 896). But for most healthy adults, the underlying chronotype is extremely difficult to shift.

Given what we already know about circadian biology, certain aspects of modern life seem perverse. Adolescents are notorious night owls, yet in some countries the school day starts so early that only the extreme outliers on the normal distribution are likely to function properly in the first lesson of the day. Is it any wonder that teachers are frequently faced with a class full of sullen, disinterested students?

In countries such as the United States and Germany, where some 20% of the working population does shift work, employers are only now starting to consider what circadian biology has to offer. Should the selection of workers for particular shifts take their chronotypes into account? Is the common practice of rotating staff between different shift rotations a recipe for industrial unrest and shoddy work?

Certainly, the measurements that have been made so far suggest that people don't readily adjust to a new working day. But we don't have firm answers to these questions, because too few studies have been done. Employers and governments are starting to recognize that more field data are needed. But moving the field of circadian biology from its current fringe position will require earmarked funding and a determined effort to raise its profile within the world's biomedical researcher agencies. At present, circadian biology remains a cross-disciplinary orphan that has few champions in the committees that decide where the grant money goes. It's time for that to change. ■

Don't create a climate of fear

US researchers studying sexual behaviour, drug use and other controversial topics need protection from political interference.

This month, 180 researchers funded by the US National Institutes of Health (NIH) started getting phone calls from the agency's headquarters in Bethesda, Maryland. The researchers were told that their names were on a list held by a Congressional committee that oversees the NIH, and that they might expect inquiries from its investigators.

The researchers concerned have done nothing wrong, but they have offended the moral sensibilities of conservative lobbyists — so they have every reason to be worried. Their 'crime' is to have convinced their scientific peers that their research into homosexuality, sexually transmitted diseases, the use of illegal drugs and other subjects is important for public health, and therefore worthy of NIH grants.

A spokesman for the House of Representatives' energy and commerce committee says that it does not plan to investigate the researchers' work formally. But the list, compiled by a religious lobbying group, has already drawn the attention of prominent Republican members of Congress. At a Congressional hearing on 2 October, they asked the NIH to explain the medical and public-health relevance of the highlighted projects. So the NIH began preparing a case to "place the research within the context of the agency's scientific mission and strategic research plan", and started calling researchers on the list to warn them that their work could be scrutinized.

This is the second time this year that the Congress has turned

a spotlight onto projects that have already been vetted by the peer-review process. In July, the House of Representatives voted by a tiny margin not to remove funding from five grants that had been singled out because they touched on such issues as female sexuality and pornography.

Every US taxpayer is entitled to hold an opinion on the merits of NIH-funded research. But not every taxpayer is an expert on the public-health needs of the United States, and neither are all of their elected representatives. This is why the government asks suitably qualified experts to recommend projects that deserve funding.

Congress has a right to ask questions about how well the peer-review process works. But applying such pointed scrutiny to individual investigators whose grants have already been funded will have a chilling effect, scaring scientists away from studying issues that may be crucial for the health and well-being of society as a whole.

These moves come at a time when the NIH is being asked whether many of its administrative functions can be outsourced to the private sector (see page 888). Federal agencies must be expected to demonstrate that they are efficiently run, but the pressures currently being brought to bear on the NIH are denting morale. If members of Congress aren't careful, they will undermine the integrity of the world's leading biomedical research agency, and ultimately threaten their constituents' health. ■





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US launches joint effort to probe dark secrets of the Universe

Tony Reichhardt, Washington

Physicists are shooting for the stars in a bid to understand the mysteries of dark energy — the enigmatic force behind the Universe's accelerating expansion.

In a ground-breaking collaboration, the US Department of Energy (DOE) has teamed up with NASA to plan a series of launches that will explore this exotic area of astrophysics.

The proposed Joint Dark Energy Mission could reach the launch pad around 2014 and, although it is not yet funded, it has strong political backing. The White House Office of Science and Technology Policy initially asked the DOE and NASA to find a way to cooperate on a space-based mission to investigate dark energy, a topic that figures prominently in both agencies' scientific 'road-maps'. A National Academy of Sciences report released last year also identified dark-energy investigations as an area where astronomers and high-energy physicists could join forces.

NASA envisages the mission as the first in a series of 'Einstein Probes', costing around \$500 million each, which would address specific problems in astrophysics (see *Nature* **420**, 593–594; 2002). Meanwhile, scientists at the DOE's Lawrence Berkeley National Laboratory in California have been leading conceptual studies of a Supernova/Accelera-



On the move: the motion of supernovae provides evidence for the existence of dark energy.

tion Probe (SNAP), which would be geared mainly towards characterizing dark energy. Saul Perlmutter, an astrophysicist at the laboratory and primary investigator on the SNAP project, says that studies of dark energy unite the interests of cosmologists and particle physicists. "The fields have become so intermixed that you really can't do one without the other," he says.

SNAP would consist of a two-metre

telescope, roughly the same diameter as the Hubble Space Telescope. But its detector would have a billion picture elements, more than 100 times the number on Hubble's best camera. From its vantage point in space, SNAP would be optimized to survey supernovae, which serve as yardsticks to calibrate the distance and speed of objects receding from us — key evidence of dark energy's mysterious pull.

Although an instrument such as SNAP would not automatically be selected for the Joint Dark Energy Mission, it would be a strong contender, says Paul Hertz, NASA's lead scientist for research on the structure and evolution of the Universe. He says that NASA and the DOE will soon form a panel to set the scientific requirements for the dark-energy mission.

The two agencies have worked together on smaller projects before, and are collaborating on the Gamma-ray Large Area Space Telescope (GLAST), which is planned for launch in 2006. The dark-energy probe would be an even closer partnership. But it would not be equal in terms of funding: Hertz reckons that NASA would pay as much as three-quarters of the cost, and the project would be based at the agency's Goddard Space Flight Center in Greenbelt, Maryland. ■

Canada prepares to pull the plug on fusion project

Geoff Brumfield

Canada's bid to host the international fusion project ITER has stalled and the country may now quit the experiment altogether.

In 2001, Canada offered a site at a nuclear facility in Clarington, Ontario, as a location for the US\$5-billion prototype fusion reactor. But it now looks certain that its bid will be allowed to die, leaving Japan, France and Spain as the remaining contenders.

Canada's federal government and the provincial government of Ontario had been prepared to guarantee US\$1.5 billion in loans to the other countries in the ITER partnership to help pay for the experiment.

They had been unwilling to stump up hard money to pay for their own bid, however.

This left supporters of the project in Canada struggling to secure federal or provincial funds before December, when a location for ITER is to be chosen.

But these efforts ground to a halt after elections earlier this month, which resulted in a change of government in Ontario, says Murray Stewart, who heads ITER Canada, the non-profit entity running the Canadian bid. Stewart says that he is launching a last-minute blitz to sell ITER to newly elected lawmakers, but the prospect of mobilizing large amounts of funding looks slim.

"I have not seen any evidence that the federal government is anxious to participate," says University of Toronto president Robert Birgeneau, who organized a government-mandated study on ITER last year.

Canada has been part of the ITER design project since 1992. But if it doesn't host the reactor, it might withdraw from ITER entirely, says David Baldwin, who heads the fusion group at nuclear-energy company General Atomics in San Diego. "There is no strong fusion community in Canada," Baldwin says, adding that such a withdrawal is unlikely to damage the project. "I don't see a threat of other pull-outs," he says. ■

Space station light show sparks aurora riddle

Tony Reichhardt, Washington

Astronaut Ed Lu returned last Monday from a six-month tour as science officer on the International Space Station with many memories and at least one nagging puzzle: what caused the mysterious flashes of light he saw while studying Earth's aurora from orbit?

Lu, who was an astrophysics researcher before becoming an astronaut in 1994, estimates that he spent 100 hours watching the northern and southern lights during his half-year in space. The auroral light

show, which takes place well below the station's 380-kilometre altitude, shimmers and pulses depending on natural variations in incoming solar particles trapped by Earth's magnetic field.

On three occasions — 11 July, 24 September and 12 October — Lu saw something markedly different: flashes as bright as the brightest stars, which lasted only a second and then blinked off again. In one instance, he called crewmate Yuri Malenchenko over to the window, and he saw the bursts too.

Lu says that they were very different from the random but harmless retinal flashes that many astronauts experience when heavy cosmic rays hit their eyeballs.

Given his limited time and ability to research the problem in space, Lu has tried to rule out other obvious explanations. The flashes didn't look like sunglint from dust particles outside the station, which rotate and last longer than a second. Nor were they meteors, which look like linear streaks. Viewing conditions were wrong for a satellite or other artificial object — the flashes only appeared in the direction of the aurora. And Lu checked weather maps, which showed no lightning storms below at the time of his observations. All of which leads him to the tentative conclusion that he was seeing a previously unreported phenomenon associated with the aurora.

Speaking to *Nature* by telephone from the space station, the astronaut said that his post-landing duties would probably keep him from attacking the problem for several months after his return. He has already had e-mail discussions with aurora specialists on the ground, however, and has yet to discover any previous satellite or astronaut report of similar optical bursts. Even though it wasn't a planned experiment, the scientist in Lu hopes to find an explanation: "It's a good thing to get out in the open, so that people who do know more can start to think about it."



Like a flash: astronaut Ed Lu's usual view of Earth's aurora (above) was punctuated by mystery flares.

Democrats condemn government 'meddling' with NIH

Erika Check, Washington

Is the fire department at the National Institutes of Health (NIH) essential to the agency's core mission? And what about its grant support staff? The Bush administration's suggestion that these and thousands of other NIH jobs might be contracted out to the private sector is drawing fire from Democrats in Congress.

"The scientific mission [of the NIH] is at risk because of a misguided privatization plan that meddles with scientists, opens the door to unnecessary security threats, and seriously undermines morale and productivity," says a 20 October letter to administration officials, signed by Representative Henry Waxman (Democrat, California) and four senators and congressmen from Maryland, where the NIH is based.

The letter was addressed to Joshua Bolten, chief of the White House budget office, and Tommy Thompson, head of the Department of Health and Human Services, to which the NIH belongs. It criticizes a government-wide initiative that asks

agencies to determine which of their jobs might be done more efficiently by the private sector, and then initiate competitions with private bidders to determine who will do a better and cheaper job. The Bush administration is committed to the competitions, but they have worried some government scientists, who say that research

agencies require skills that are not available from outside (see *Nature* 424, 478; 2003).

Waxman's letter says the competitions are draining resources and hampering the NIH's core mission. It adds that the agency is spending \$15 million on job reviews, and that NIH employees have already spent 100,000 hours preparing for competitions. The agency also plans to put some 4,000 jobs — including some scientific roles — up for bidding by 2005, the letter claims.

Health department spokesman Bill Pierce says Thompson is merely trying to ensure that the government saves as much money as possible. "Very few people would say it would not be a worthy goal for the NIH to do things more efficiently," Pierce adds.

Pierce says the government has to ensure that federal dollars are well spent. The NIH, Pierce notes, has won both competitions held this year, and will therefore retain its grant-support staff and infrastructure managers. "For the NIH to be doing its job better and more efficiently, but still within the NIH, is very positive," he says.



South Florida rocked as dean quits over political funding



Beleaguered: the University of South Florida's medical college has been hit by resignations.

Rex Dalton, San Diego

Plans by the University of South Florida (USF) to enter the top league of US biomedical research institutions suffered another blow this month with the resignation of the dean of its medical school.

Robert Daugherty, who ran three medical colleges at the university, resigned on 13 October after admitting that he was raising money from faculty members to support a Florida state politician. The politician in question, Johnnie Byrd, has been instrumental in directing state funds towards the Tampa-based university.

The resignation is the latest in a series of setbacks for USF's expansion plans, which are part of a wider push by institutions in the fourth most populous US state to punch their weight in biomedical research. Earlier this year, Michael Mullan, a prominent neuroscientist, resigned from USF after being accused of inappropriate behaviour with female staff. The resignation pushed a wealthy Alzheimer's research centre, led by Mullan, off the campus — along with a major federal grant to build a laboratory to study the genetic causes of drug addiction.

Mullan, who was a member of the team that discovered a gene linked to Alzheimer's disease (M. C. Chartier-Harlin *et al.* *Nature* **353**, 844–846; 1991), was recruited by USF from Imperial College London in 1992. He resigned after a USF investigation concluded that he had sexually harassed one woman and created

“a serious risk” of violating university policies in his pursuit of personal relationships with women in his lab.

The 25-page report quotes witnesses as saying that he ran his lab in a controlling manner like “a cult” — allegedly favouring those women with whom he was pursuing personal relationships. He allegedly threatened the career of one woman after their intimate relationship ceased, the report says.

In an interview, Mullan emphatically denied any impropriety. He called the USF report “absolute rubbish” and said that it was part of a plan by top officials to smear him.

After resigning in January, Mullan filed a civil lawsuit in Tampa against a former USF researcher whom he allegedly harassed in 1997. Mullan claims that Karen Gosche defamed him by commenting anonymously in a Tampa newspaper article about the USF report. On 13 October, a state judge rejected USF's request to dismiss this lawsuit, a move that may open the way for court-ordered testimony from women witnesses in the university's report.

USF attorney, Tom Gonzales, who represents Gosche, says that Mullan's lawsuit is an attempt to extract “revenge” on the university. Gosche, who is now a contract neuroscientist in Florida, says that USF produced an accurate and appropriate report. But she adds that she is disturbed at being legally threatened for cooperating with the inquiry.

The Alzheimer's institute headed by Mullan at USF left the campus with him. The Roskamp Institute is now based 30 km away in Sarasota, home of Robert Roskamp, the businessman and USF benefactor who contributed \$5 million to set up the centre. A laboratory to study genetic factors in drug addiction, to be supported by \$5.85 million from the White House Office of National Drug Control Policy, has also moved to Sarasota instead of to USF as originally planned.

Meanwhile, USF officials have been trying to establish a new Alzheimer's research institute. Byrd, the speaker of the Florida House of Representatives, last year helped to pass state legislation to provide \$25 million for this project, and he is supporting a proposal to provide another \$45 million for it next year.

But USF president Judy Genshaft demanded Daugherty's resignation after learning that he was trying to collect political contributions for Byrd from university employees. Daugherty couldn't be reached for comment; Byrd denies knowing of the fund-raising effort. ■



Michael Mullan resigned in January.



Count me in: a new species of scorpionfish joins over 15,000 fish already identified.

Marine survey sees net gain in number of fish species

Betsy Mason, Washington

There are some 20,000 different species of fish in the world's seas and oceans, about a quarter of which are still to be discovered and classified, says the first report of a census of marine life.

More than 300 scientists from 53 countries are working on the Census of Marine Life (COML), a ten-year, \$1-billion project due to be completed in 2010. Some 210,000 species of marine life have already been documented by the project, and more than 2 million are likely to be recorded eventually.

“New species are being discovered at the rate of about three a week,” says Ron O'Dor of Dalhousie University, Canada, senior scientist for the COML. “Most of these species are coming from areas that haven't previously been explored.”

The initiative is benefiting from technologies that allow scientists to explore new places and to see tiny or evasive creatures for the first time. “Every time a new technology is developed or an existing technology is refined, it opens up a new window on the ocean,” says David Farmer of the University of Rhode Island, COML's lead scientist for technology.

The development of remotely operated submarines allows scientists to explore deeper and colder waters, such as the polar oceans and the Mid-Atlantic Ridge, and better sonar helps them to locate, track and even identify different fish species. The scientists are also using computer chips as electronic fish tags to track the movement of fish and record information such as heart rate and temperature.

In addition, researchers are using short sequences of DNA to help identify and catalogue species, particularly smaller ones such as plankton. “We hope that part of the census will include a DNA barcode for each new species,” says Jesse Ausubel of Rockefeller University in New York. “This is something that would have been impossible just five years ago.” ■

Biodiversity schemes take root in China

David Cyranoski

For years, environmentalists have watched aghast as huge farming and construction projects have threatened to wipe out swaths of China's ecology. But as modernization marches westwards across the country, scientists are proposing initiatives to help conserve a region of exceptional biodiversity.

On 24 October, researchers from the Chinese Academy of Sciences (CAS) Institute of Botany in Kunming met with government officials to negotiate funding for a national germplasm bank to be based at the institute. They are now optimistic that they will obtain 148 million renminbi (US\$18 million) for the bank, plans for which passed a government-sponsored scientific evaluation in early September.

And on 27 October, the CAS Kunming Institute of Zoology hosted a conference to launch a collaboration between the CAS, Germany's Max Planck Society and the University of Chicago to train China's next generation of conservation biologists.

The two initiatives add momentum to the expansion of China's nature reserves, which have doubled in number in the past five years and now occupy 16% of its land area, says Zhigang Jiang, a zoologist at the CAS Institute of Zoology in Beijing and director of China's Endangered Species Scientific Commission. The reserves are concentrated in western and southwestern regions of the country.

The latest schemes will seek to protect some of the world's most ecologically diverse regions, says botanist DeZhu Li, deputy



Species such as the endangered *Davidia* tree (inset) face severe ecological pressures in southwestern China.

director of the Kunming botanical institute.

Ecologists have long been concerned for southwestern China's endangered species — such as the *Davidia* tree and Yunnan snub-nose monkey — arguing that they are under threat from agricultural and industrial development in the region. Five years ago, Zhengyi Wu, a former director of the botanical institute, brought the matter directly to the attention of then Chinese prime minister Zhu Rongji. "Scientists called on Zhu to take steps before industrialization destroyed the environment," says Li. "We told him that before any major industrial change, protection of the environment is urgent."

Zhu's support, before his retirement last March, led to this month's negotiations on the germplasm bank, which would store seeds from thousands of plants and samples from

microorganisms native to the south-western regions of Yunnan, Guizhou, Sichuan, Guansi and Tibet. The bank will also support *in vitro* fertilization and cryogenic preservation of plants.

The project is being watched closely by ecologists outside China. Hugh Pritchard, head of seed conservation at Britain's Millennium Seed Bank Project based at the Royal Botanic Gardens in Kew, says: "This is the only other serious attempt at mass conservation of plant biodiversity that I know of."

Li says that the Chinese group plans to collect 4,000 seeds over the next five years, eventually rising to 19,000. Pritchard adds that many samples will also be deposited with the British project, helping it towards its goal of collecting 24,000 species by 2010. The Chinese effort will be a useful ally to his team's project, Pritchard says.

But a lack of trained professionals might hamper China's conservation ideals. "Much of this biodiversity has never been handled before," says Pritchard. He says that the project will throw up some interesting problems, such as how to differentiate between seeds that are no longer viable and those that are just taking a long time to germinate.

Chung-I Wu, an evolutionary geneticist at the University of Chicago who helped to organize the 27 October meeting, says that China needs a new generation of biologists to implement its conservation plans. "For conservation biology to have a future, it must have a sound scientific basis," he argues. "It will need to attract those who might otherwise become the brightest computer scientists." ■

Europe kills cash flow to EURESCO science meetings

Quirin Schiermeier, Munich

A popular series of intimate, cutting-edge scientific meetings for European researchers is facing the axe because it doesn't fit the priorities of the European Union (EU) Framework programme of research.

The European Research Conferences (EURESCO) were started in 1990 as Europe's answer to the Gordon Research Conferences, which attract top researchers from all over the world to discuss specific scientific topics, but usually take place in the United States.

The EU's Sixth Framework Programme, which has allocated €40 million (US\$47 million) over four years for conferences and training courses, gives priority to helping young researchers attend large meetings or small events with a strong element of training, such as summer schools. As a result, all of this year's applications for grants to support

EURESCO meetings have been turned down.

Some 40,000 scientists have attended more than 490 EURESCO meetings, which are organized by the Strasbourg-based European Science Foundation (ESF). Most pay to attend, but extra money is needed for travel and accommodation for speakers. Gordon conferences get this money from corporate sponsorship, but the EURESCO meetings have relied on EU grants.

Now competition for such grants is intense, says Nicholas Deliyakis, a project officer at the European Commission's research directorate. Only 10% of all proposed events have been selected for funding under the Sixth Framework Programme. Deliyakis says that although EURESCO meetings are not explicitly excluded, they are unlikely to be supported.

"EURESCO meetings were always

absolute highlights — short, intensive and immensely productive," says Thomas Stocker, a climate researcher at the University of Bern in Switzerland.

"I would be very sad if EURESCO meetings were to be abandoned," agrees Stig Stenholm, a quantum physicist at the Royal Institute of Technology in Stockholm, Sweden. "In my field, they have substantially helped to forge a strong European research community."

Seventeen EURESCO meetings will still be held next year, with a further six in 2005, on the basis of grants already approved under the previous Framework programme. After that, the ESF is exploring options for continuing the series. "We are very keen for these conferences to be re-established," says Enric Banda, the ESF's secretary general. ■

♦ www.esf.org/esf_euresco_home.php?language=0

Doubts swirl around plan to use rigs as reefs

Betsy Mason, Washington

Offshore oil platforms could be allowed to remain in place as artificial habitats for marine life, under legislation before the US Congress.

The planned law is being backed by oil companies — who stand to save hundreds of millions of dollars in decommissioning costs — and by local fishermen. Its impact would be felt mainly in the Gulf of Mexico. But environmentalists see the law as little more than a ruse to conserve the companies' cash. Ecologists are unsure of the idea's merits, but some say that there is no real evidence to support it.

Current federal law requires companies to remove platforms within a year after the oil stops flowing, and to leave the sites as they found them. But the Rigs to Reefs Act of 2003, which is sponsored by Representative David Vitter (Republican, Louisiana), would allow platforms to be left in place as artificial reefs to provide habitat for coral, fish and other species. There are more than 4,000 offshore platforms in the gulf, with around 100 being decommissioned every year.

"Offshore oil and gas platforms are home to some of the most prolific ecosystems on our planet," Vitter told a subcommittee of the House Resources Committee on 17 September, when he introduced the bill.

No one disputes the fact that the rigs lead to local concentrations of wildlife, and the idea is often touted as a 'win-win' proposition that would benefit both business and the environment. But some ecologists think that, instead of providing a habitat for the growth of new fish, the platforms merely attract fish from elsewhere, making the fish easier to catch and putting even more pressure on overfished species such as red snapper. Catching them is, as Vivian Newman of environmental organization the Sierra Club puts it, "like shooting fish in a barrel".

Scientists studying the ecology of the gulf agree that the question of attraction versus production is a critical one, but are divided over the answer. Robert Shipp, a fisheries scientist at the University of South Alabama in Mobile, has studied the effects of artificial reefs on red snapper populations for 15 years, and is convinced that, since the oil industry began building platforms almost 60 years ago, the gulf's snapper population has rebounded.

Alabama has allowed recreational fishermen to create around 20,000 artificial reefs off its coast, constructed primarily of specially shaped cement pyramids and domes. This has transformed its 1,200-square-mile area from habitat favouring less economically valuable species, such as flatfish and porgies, to one where snapper and grouper thrive. "It's changing the nature of the biomass," says Shipp. "If you have more reefs, you will



Ecologists are unsure whether artificial reefs are a help or a hindrance in fish conservation.



Climb the rigging: populations of endangered fish can surge around disused oil platforms.

have more reef species, and those are the valuable ones."

But fisheries scientist Charles Wilson of Louisiana State University in Baton Rouge believes that the red snapper population may still be in trouble. The fish on the artificial reefs in Alabama are smaller and are reproducing earlier than those in Louisiana, he says: "This could be a sign of excessive fishing pressure." As fishermen keep catching the adults, the population responds by reproducing at a younger age. Other fisheries have shown this effect before collapsing from overfishing, he says.

And attracting fish to artificial reefs could pose another problem, according to marine ecologist Felicia Coleman of Florida State University in Tallahassee. Species such as grouper have evolved to spawn in particular areas of the gulf so that their young are transported by currents to near-shore nurseries. But because hard substrate is so scarce in the mud-dominated gulf, the fish could be

attracted to platforms where currents will send the spawn further offshore to die, instead of towards the shore. "As far as platforms contributing to productivity of fish populations are concerned, I'd say the jury is still out," Coleman says.

States such as Texas and Louisiana already have rigs-to-reefs programmes for state-controlled coastal waters, under which oil companies give the states half of the money saved, to help with maintenance. But issues of liability have limited the programmes' popularity, and only around 100 platforms have been donated thus far. The proposed law would relieve the oil companies of liability for any damage caused by the rigs and allow them to be left in federal waters, further offshore, increasing savings to the industry.

Although the proposed Rigs to Reefs Act is aimed primarily at the Gulf of Mexico, the same approach may also be used in California, where a bill allowing the practice was passed by the state legislature in 2001, but vetoed by then governor Gray Davis. That bill could be revived and signed by the state's new governor, Arnold Schwarzenegger, environmental groups say.

But the ecological picture is murkier in California than in the Gulf of Mexico, because many natural reefs already exist. Fish ecologist Mark Carr of the University of California, Santa Cruz, has been comparing species and numbers of fish at natural and artificial reefs. He has found that the story varies for each species and for each reef.

"To really identify the ecological consequence of artificial reefs, we need to know how well the fish grow, survive and reproduce, and what their relative fates are," says Carr. "We just don't know yet."

Arctic clean-up project aims to confront Soviet pollution legacy

London The environmental legacies of the cold-war era in the Arctic are set to be tackled, thanks to a multimillion-dollar fund devoted to the cause.

The four-year plan, which has a fund of more than US\$30 million, will assess ten hotspots of environmental problems in the Russian Arctic. These include some of Russia's most polluted cities, which have been affected by industries established under the former Soviet Union, such as oil and mineral mining. The project will also target old military marine sites where submarines have been abandoned and left to rust away.

Two bodies — the United Nations Environment Programme and the Advisory Committee on Protection of the Sea, a London-based environmental group — will determine the cost of cleaning up the sites, and will test technologies such as the use of marine algae that absorb oil.

The project's backers, including several Arctic nations and the Global Environment Facility, an international environment fund based in Washington DC, hope the studies will lead to a full-scale environmental restoration programme.



Out in the cold: Russian submarines left to rot.

Foreign students to foot the bill for US security scheme

Washington The US government is setting up a system to keep tabs on foreigners studying in the country — and it expects the students themselves to foot the bill.

The Department of Homeland Security, which oversees immigration, proposed last week that students seeking US visas be charged a \$100 fee. Homeland Security undersecretary Asa Hutchinson says that he expects the scheme to raise more than \$30 million a year, which will go towards a computerized tracking system designed to seek out terrorists posing as students.

Since the attacks of 11 September 2001, immigration rules have hampered students hoping to study in the United States, and academic bodies are fearful that the fee will

Wolves under threat as rabies outbreak bites

London An outbreak of rabies has hit one of the world's rarest dogs, the Ethiopian wolf (*Canis simensis*, pictured). Twenty of the animals have died in the Bale Mountains, in the southeast of the country, over the past few weeks — a severe blow for a species that numbers only 500 wolves overall. Three-quarters of the wolf population died during a similar outbreak in 1991. Conservation biologist Claudio Sillero of the University of Oxford, UK, says that a similar proportion could die this time around. Conservationists think that the wolves caught the disease from domestic dogs — although dogs in the region have been vaccinated. Wildlife managers have long wanted to vaccinate the wolves themselves, but Ethiopian authorities have so far refused, citing concerns over the potential side-effects of such a move. "We could arguably have prevented the current outbreak," says Sillero.



be yet another impediment. Students will probably have to pay in US currency or by cheque or credit card — but such means are not always available to students in developing countries.

US beefs up policing of transgenic crop trials

San Francisco The US Department of Agriculture (USDA) is establishing a unit to monitor field trials of genetically modified crops.

Transgenic crops have been field-tested, grown and eaten in the United States for years. But crops that are genetically tweaked to make drugs or industrial compounds have faced more opposition. In 2002, ProdiGene of College Station, Texas, was fined \$250,000 for mismanaging a trial of maize engineered to produce a pig vaccine. Large quantities of maize and soy beans in Iowa and Nebraska had to be destroyed after possible contamination by the transgenic maize.

This summer, the USDA began requiring specific permits for the testing of non-food biotech crops; before this, companies merely had to notify the agency that tests were taking place. To enforce the rule, the USDA announced on 17 October that it is to form a compliance unit within its Biotechnology Regulatory Services programme dedicated to inspections, raising the number of services staff from 33 to 50.

Tropical climate study gives view of global heatwave

Munich A study of the tropical Pacific Ocean has given climate researchers a global picture of how temperatures changed during an exceptionally rapid period of warming 55 million years ago.

Researchers think that this spike of warming, called the Palaeocene/Eocene

Thermal Maximum, was caused by a belch of methane from reservoirs in the sea floor (see *Nature* 423, 681–682; 2003). Researchers knew that at high latitudes, sea surface temperatures rose by some 10 °C over a period of 10,000 years — they deduced this from the ratios of different isotopes in the preserved shells of marine organisms. But no one had been able to find suitable samples of such shells for this brief time period in the tropics.

Now James Zachos of the University of California, Santa Cruz, and his colleagues have managed to analyse organisms in a tropical seafloor sample, and found that these waters warmed by about 5 °C (J. C. Zachos *et al.* *Science* doi:10.1126/science.1090110; 2003). Their findings are consistent with climate models used to describe such periods of rapid temperature change.

NASA turns to sub firm in nuclear power play

Washington A naval office that is more at home designing nuclear reactors for submarines may soon adopt its technologies for outer space.

NASA is negotiating with the Department of Energy to hire Naval Reactors, based in Arlington, Virginia, to design the power source for its ambitious nuclear-powered Jupiter Icy Moons Orbiter (JIMO), planned for launch after 2011.

Naval Reactors has a reputation for quality and safety, says Alan Newhouse, manager of NASA's nuclear programme, called Project Prometheus. But it will probably need outside advice in adapting its nuclear technology for space. The deal has not yet been signed, but Admiral Frank Bowman, head of the Naval Nuclear Propulsion Programme, said last week that he supports the project.

Fertilized to death

Vast quantities of nitrogen being poured onto farmers' fields are wreaking havoc with our forests. Nicola Nosengo investigates.

T. PAGE/ECOSCENE

Dotted throughout forests around the world, yellowed leaves and thinning crowns suggest that some trees are dying an early death. But the culprit may come as something of a surprise. It isn't just pollution spewed from car fumes, or damage from insects proliferating thanks to global warming. Our forests are facing a quieter villain. They're being plagued by the very stuff that has provided people with food for the past hundred years — fertilizer.

The use of fertilizer changed dramatically in the twentieth century. In the late 1890s, people struggled to get enough fertilizer for their fields — the main sources were bird guano from the Pacific islands and saltpetre from the deserts of Chile. But as the world's population grew, it became clear that we would need a cheaper, easier way to get a usable form of nitrogen. That problem was solved in 1909 by Fritz Haber and Carl Bosch, who devised the first industrial process to turn nitrogen gas (N_2) in the air into ammonia (NH_3). The result was a ready supply of cheap fertilizer, which has powered global food production ever since¹.

But that success story is now becoming an environmental scourge. Unused fertilizer is washing off fields into rivers, poisoning coastal waters and causing acid rain. Scientists are worried that this flood of food could be causing the slow — and possibly irreversible — death of our forests.

Nitrogen is a relatively unreactive gas. But it can spawn a range of reactive molecules thanks to the work of certain bacteria, the burning of fossil fuel or the manufacture of fertilizer. Collectively known as reactive nitrogen, this family of molecules includes ammonia, nitrate ions (NO_3^-) and nitrogen oxide gases (NO_x), and its production has more than doubled over the past century.

Food for thought

Until the early 1900s, most reactive nitrogen was produced by bacteria and amounted to about 100 million tonnes per year. But human activity alone now generates more than 160 million tonnes per year (ref. 2) — 25 million tonnes through burning fossil fuels, mostly in cars, and more than 100 million tonnes from the industrial production of fertilizer. If the current rate of increase continues, global production of reactive nitrogen is predicted to reach between 250 million and 900 million tonnes per year by 2100.

The trouble is that a lot of this nitrogen doesn't end up where it is meant to be — in our food. James Galloway, an environmental



scientist at the University of Virginia in Charlottesville, estimates that almost half of the nitrogen spread onto fields is not taken up by crops but instead washes away.

Most of it leaks through the soil into groundwater as nitrate, which then washes into ponds or coastal waters. There the excess nutrient fuels the rampant growth of algae, which in turn uses much of the oxygen in the water, suffocating fish and other marine life. The agricultural runoff down the Mississippi river is so packed with nitrogen and other nutrients that there is now a giant patch of algae covering 20,000 square kilometres in the Gulf of Mexico. Throughout the United States, one-third of the coastal rivers and bays show similar effects on a smaller scale².

Less commonly, excess nitrate can end up in drinking water, where it can cause 'blue baby' syndrome, or methaemoglobinaemia. In this rare but sometimes fatal condition, red blood cells are no longer able to perform their vital role of carrying oxygen around the body, which subsequently turns an infant's lips an oxygen-deprived blue.

Such effects on coastal and human health have grabbed the headlines and public attention, but scientists are now concerned about more subtle events. A significant amount of reactive nitrogen ends up in the air as ammonia and NO_x where it increases the amount of low-level ozone, which in turn contributes to smog and global warming. In the atmosphere, some NO_x dissolves in water vapour to produce nitric acid, which falls back to the ground as acid rain. The ammonia, although it is alkaline, can also make soils more acidic — as microbes digest the ammonia they produce nitrate and acidic hydrogen ions.

Much of this reactive nitrogen is falling on our forests, where the results can already be seen³. Trees are dying and the relative abundance of different plant species in some woods has started to change⁴. "The effect on forests

is slower and less visible than on coastal environments," says Galloway. But that could mean that once effects become obvious, it may be too late for the trees to recover.

In the late 1980s, John Aber of the University of New Hampshire in Durham described how a forest might react to a nitrogen overdose⁵. He suggested that a forest doused with nitrogen initially thrives, but at some point, input of nitrogen exceeds demand. As plants are no longer able to absorb it, the nitrogen builds up in the soil, mostly as nitrates. These negatively charged ions attract positively charged ions such as calcium and magnesium, and carry them into the water table. This deprives the trees of fundamental nutrients just as their demand for them is growing. Weakened, the trees become increasingly vulnerable to frost, drought and parasites. At the same time, rising soil acidity causes a loss in biodiversity in the undergrowth.

Poisoned land

Confirming Aber's hypothesis and predicting the likely course of future events is a tricky proposition. To that end, researchers at the IVL Swedish Environmental Research Institute in Gothenburg have been studying nitrogen saturation in the Gårdsjön forest in southeastern Sweden since 1991. Part of the forest is covered with a transparent roof, watered with clean water and acts as a control site. In another section, researchers have been adding 40 kg of nitrogen per hectare per year to a site that used to get less than 10 kg per hectare from atmospheric depositions — an unpolluted forest should get less than 5 kg per hectare per year. The massive overdose speeds up the process of nitrogen poisoning, giving scientists an idea of what might occur in the future. "Not very much happened for the first five years," says Filip Moldan of the Swedish Environmental Research Institute, who coordinates the project. "But since then

J.L. AMOS/CORBIS

Mixed blessing: the face of farming has been transformed by the industrial production of nitrogen-based fertilizer, shown here being spread on a field (opposite, far left) and loaded into a rail car. But overuse has led to suffocating algal blooms in rivers (left) and damage to ecosystems caused by acid rain (below).



W. & D. MCINTYRE/CORBIS

we are seeing changes wherever we look.”

The over-fertilized trees are now growing faster than normal, and the levels of various nutrients in the foliage have changed — as predicted by Aber, the leaves contain more nitrogen, and less calcium and magnesium than in normal trees. And about 10% of the added nitrogen is now leaking out of the forest as nitrate in groundwater, says Moldan. He hopes to keep the experiment running for another decade to see how the saturation process will proceed. “At some point the soil will probably become unable to retain any of the nitrogen added, and the forest will start to decline,” he predicts. “But we don’t know how long this will take.”

Nor is it clear how different types of forests will respond. Although there isn’t much information to go on, studies suggest that humid tropical forests will reach nitrogen saturation more quickly than those in temperate climes⁶. Some tree species, such as sugar maple and red spruce, seem to be particularly sensitive to additional nitrogen and could disappear completely from some sites.

So how can we save our forests? Many European countries have tried to counteract the build-up of reactive nitrogen by liming — adding calcium or magnesium carbonates to the soil. That reduces the soil’s acidity and adds back nutrients. But the process is too expensive to use on a large scale. And over-liming would kill soil microbes, again altering the ecosystem. “Liming only cures the symptoms, not the disease,” says Aber.

A few tentative steps were taken to address the problem some 30 years ago. In the 1970s, both sulphur and nitrogen were flagged up as the source of acid rain. Initiatives — such as a variety of national laws including the 1970 Clean Air Act in the United States — tried to limit both sulphur oxides and NO_x. But the NO_x provisions only controlled the amount produced by individual cars, largely ignoring

agricultural contributions. Only the sulphur controls were truly successful. “Sulphur deposition to soil in Europe and North America has decreased up to 70%, but nitrogen deposition is constant or slightly increasing,” says Aber.

Cutting back

More recently, 28 European countries signed the 1999 Gothenburg protocol, one of the goals of which is to reduce emissions of NO_x by 41% and ammonia by 17% by 2010, compared with emissions in 1990. As of 2002 they seemed to be on track: emissions of NO_x and ammonia were down 23% and 6%, respectively. But that is mainly thanks to controls on fossil-fuel burning in Germany, Britain and the Netherlands, which won’t be able to reduce emissions much further, says Moldan. Meanwhile emissions from some



A plastic canopy protects trees in Gårdsjön, as part of an experiment to test the effects of nitrogen.

countries in Europe, as well as China and Russia, are rapidly increasing.

Scientists are sceptical about how effective the protocol will prove to be, particularly as it fails to address how agriculture can reduce nitrogen emissions in the face of a growing population and its need for food. “Countries such as China have no intention of reducing their use of nitrogen,” says Moldan. “In fact they are firmly committed to increasing it.”

Concerned scientists will meet next October in Nanjing, China, at the 3rd International Nitrogen Conference. Their aim is to propose a ‘Nanjing protocol’ to address the issue of nitrogen at a global level. According to Galloway, one of the meeting’s promoters, the protocol should include not only limitations on NO_x and ammonia emissions, but should focus on an integrated approach to managing reactive nitrogen.

The point, he says, is to make nitrogen use in farming more efficient. Nitrogen can be recycled from crop waste, manure and slaughtered animals, either by turning it back into gaseous N₂ or into animal feed⁷. Farmers could also use less fertilizer, if there were ways to cut usage without reducing crop yields. All of these ideas are technically feasible, says Galloway, but are so expensive that no one currently bothers. That needs to change. “We cannot replace reactive nitrogen as we did with chlorofluorocarbons in refrigerators,” says Galloway. “We need it and we will need it more in the future, there is no way around that.” ■

Nicola Nosengo was, until recently, an intern in Nature’s Munich office.

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Restless nights, listless days

More and more people's working and social lives are blighted by skewed sleep patterns. Is it time for the medical mainstream to take notice of what neuroscientists are learning about the body clock? Alison Abbott reports.

As thousands of researchers from Europe and Asia join their American colleagues in New Orleans next week for the annual Society for Neuroscience conference, much of the café talk will revolve around jetlag and the merits of melatonin. Does taking this hormone really help us to adjust more quickly to a new time zone?

At least neuroscientists should be able to get an informed opinion. Their discipline includes specialists who study the body's daily, or circadian, rhythms. And the good news, from a recent analysis of the few studies that have investigated the effects of melatonin, is that it does help to reset weary travellers' body clocks¹. The hormone is secreted from the brain's pineal gland during darkness, but levels fall when day breaks. It influences the body's 'master clock' — the suprachiasmatic nuclei (SCN) — located in a region of the brain called the hypothalamus. So by taking extra melatonin at the desired bedtime, you can retrain your clock more quickly to match the local time zone.

Jetlag is just one example of a disruption to circadian rhythms. Your body clock can be upset by disease, seasonal changes in day length, and even ageing — causing sleep patterns to shift from the norm. You can also run into problems if you try to ignore your biological rhythms, for example when working night shifts. But circadian biologists are finding that broken body clocks can, in some cases, be mended. They are now calling on governments to recognize the societal importance of their work, and to provide the funding needed to move the field from its current fringe status into the mainstream of medicine.

When your biological clock is running smoothly, body temperature and the levels of various hormones, such as cortisol, peak and trough in fixed cycles, synchronized to the onset of night and day (see diagram, opposite). Body temperature reaches a maximum in the early evening and then falls, generally dropping by a full degree centigrade by the end of a night's sleep. Many other physiological functions show similarly resilient

rhythms. What's more, the basic tempo of the cycle is fixed — it can't get much faster or slower — even in the absence of external cues that signal the time of day.

The workings of circadian clocks are gradually being revealed, right down to their molecular nuts and bolts. Central to the timekeeping mechanism are the SCN, which comprise two clusters of 50,000 neurons, one on each side of the brain. A dozen or so time-keeping genes — with names such as *Period*, *Clock* and *Cryptochrome* — have been identified in the human SCN over the past few years. Together, they orchestrate the daily cycle of sleep and wakefulness. The genes are periodically switched on and off by complex feedback loops that are influenced by our exposure to light, which the SCN seem to sense through specialized neurons called melanopsin cells in the eye's retina. These are sensitive to blue light, but have nothing to do with vision².

Circadian biologists are now beginning to understand what can go wrong with this system, and are figuring out how to put it

thing, but coping with the loss of active neurons from the SCN, as occurs in Alzheimer's disease for example⁶, is more challenging. The internal clock of an Alzheimer's patient may be out of synch with the actual cycle of day and night by several hours. "Normal old people, and old people with other types of dementia, hit their lowest body temperatures between 3 and 5 a.m.," says Harper, who has compared the circadian rhythms of patients with different dementias⁷. "But those with Alzheimer's frequently occur much later — even as late as noon."

Night nursing

So Alzheimer's sufferers tend to be active when those who are caring for them want to sleep. "In fact, most Alzheimer's patients go into nursing homes not because of their memory disorders, but because families can't cope when they wander around at night," says Eus Van Someren of the Netherlands Institute of Brain Research in Amsterdam. He is on the verge of completing the first study to follow the progress of Alzheimer's patients exposed to different light conditions.

Earlier studies⁸ at the Amsterdam institute showed that exposing elderly rats — suffering from loss of active SCN neurons and disturbed sleep patterns — to bright light restored both healthier sleep patterns and the number of active SCN neurons. "The neurons become inactive but don't disappear, and at least in the rat they can be reactivated," says Van Someren. "So we wondered if this 'use-it-or-lose-it' phenomenon could happen also in old and demented people."

In 1999, Van Someren and his colleagues began their long-term study of how light conditions affect Alzheimer's patients. It has involved 190 subjects in 12 Dutch residential homes installed with light boxes in the ceilings to mimic skylights. In half of the homes these remained bright during the day, whereas in control homes the lighting was kept low. The researchers measured the strength of the patients' biological rhythms every six months, and monitored their sleep over a two-week period using an activity monitor strapped to the wrist.

Van Someren is still analysing the data generated during the first three-and-a-half years of the study, but he reported his early findings to the 1st World Congress of Chronobiology in Sapporo, Japan, in September. Bright lighting during the day shifted circadian rhythms back towards a normal pattern, improving sleep patterns and mood. It also seemed to slow the decline in the patients' cognitive capacity.

Schizophrenics are similarly often active at night. "Psychiatrists have tended to dismiss this observation with the notion that 'they can't hold down a job, so of course they will be staying up all night,'" says Russell

Tired and troubled: sleep researchers hope that better knowledge of the body clock's workings — and of how to influence its rhythm — will help those struggling with drowsy days and never-ending nights.

right. Studies of the newly identified clock genes are already helping to reveal the causes of diseases that result in extremely unusual patterns of sleep and wakefulness (see 'Seriously out of synch', overleaf). And experts are now experimenting with light or melatonin as a fix for various circadian disruptions. For instance, Charles Czeisler, a sleep researcher at the Brigham and Women's Hospital in Boston, is testing blue light³ for resetting melatonin rhythms.

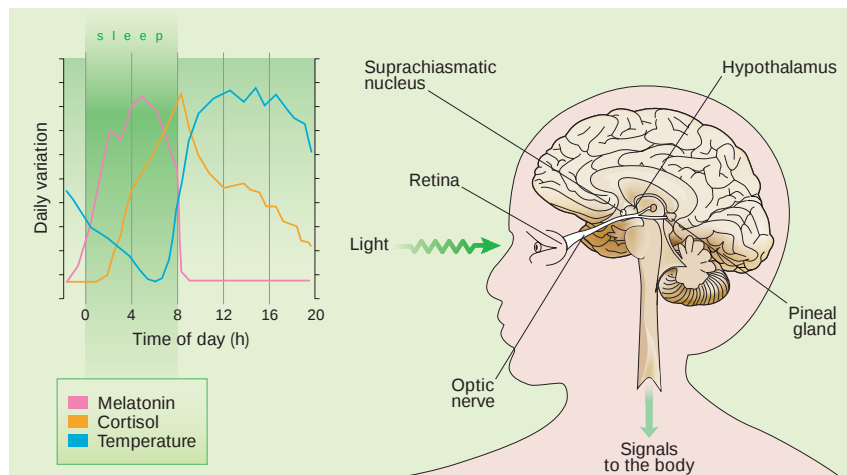
Cycle paths

Although everyone's rhythms operate on a daily cycle, our clocks can be set very differently, causing different people to be most active and alert at different times of the day. In general, we drift towards 'eveningness' during adolescence, and towards 'morningness' in old age. But our basic personal preferences are resilient: 'larks' who wake at

6 a.m. and 'owls' who never retire before 1 a.m. will remain as such, whatever the pressure from parents, spouses or employers⁴.

As well as being up with the larks, elderly people produce less melatonin overall, and therefore tend to have more disturbed sleep. But a team led by Kazuo Mishima, a sleep researcher at the National Center of Neurology and Psychiatry in Ichikawa, Japan, has shown that exposure to bright light in the middle of the day can increase night-time melatonin production and promote deep sleep for many old people⁵. "In general, the more light old people have, the stronger their daily rhythms will be — and so their sleep patterns improve," says David Harper, a specialist in geriatric psychiatry at the McLean Hospital in Belmont, Massachusetts. "It is unfortunate that the old often live in poorly lit homes and don't get out much."

Adapting to normal signs of ageing is one



Foster, a circadian biologist at Imperial College, London. Foster and his colleague Katharina Wulff are now trying to find out whether schizophrenics have disrupted circadian rhythms. The eight individuals they have so far studied tended towards extreme eveningness, and the researchers are now applying to the UK Medical Research Council's new Brain Sciences Initiative for funding to study the effect of treating schizophrenics with light. "It will be interesting to see if consolidating sleep-wake profiles helps to control schizophrenic episodes," says Foster.

Light also helps to relieve the symptoms of depression that afflict patients with seasonal affective disorder. Also called the 'winter blues', the problem is clearly linked to disrupted circadian rhythms as the nights draw in⁹. "Light-treatment results can be seen within a week — an advantage over classical antidepressants, which take several weeks to kick in", says Anna Wirz-Justice, a circadian biologist at the University of Basel. In Switzerland, at least, light therapy is paid for by medical insurance companies.

While scientists are finding that light or melatonin can help when daily biological rhythms are off-balance, they are also discovering the alarming extent to which modern society works against, rather than with, normal circadian biology. Employers, educators and politicians are only now realizing that there may be a serious issue to face.

Shift work is the most notorious example. Night-shift workers do not adjust their circadian rhythms because they are exposed



Out of the blue: doses of light therapy could boost production of hormones that set the body clock.

to daylight, particularly in summer, before and after their shifts. Small wonder, then, that productivity is lowest during night shifts. "So far, industry has not taken this seriously enough," says Till Roenneberg, a circadian biologist at the University of Munich, Germany.

Driving progress

Roenneberg is running the first large study — sponsored by the car manufacturer Volkswagen — to relate individual preferences for morningness and eveningness to physiology and performance under different factory light conditions. His team began by fitting some areas of Volkswagen's Wolfsburg plant with brighter lighting than usual, and then examined workers under controlled laboratory conditions before and after this intervention. The study, involving around 100

workers, will be completed by next spring. "Volkswagen is in the vanguard, but other car companies are now showing a lot of interest in researching the night-shift problem," says Roenneberg.

Students would also benefit if greater attention were paid to their circadian biology. Roenneberg irritated local politicians last year by arguing publicly against early starts for school students — most German school days begin at 8 a.m., and some at 7 a.m. "We know that adolescents are shifted towards eveningness and are just not very alert early in the morning," he argues. In the United States, a bill has been introduced in the Congress proposing to reward schools that elect to delay starting times with grants of up to \$25,000.

Around the world, awareness of the social importance of circadian biology is slowly growing. Foster, for example, has been asked to discuss the subject with British Prime Minister Tony Blair. "The huge impact of circadian biology on health and wealth is starting to be appreciated by politicians," he says. In Japan, scientists are planning studies on the effects that brightly lit 24-hour shopping malls and late-night gaming arcades are having on daytime performance, particularly among the young.

Nevertheless, circadian biologists say that investment in their field lags behind that in disciplines with similar societal relevance. It is time, they suggest, for politicians and funding agencies to see the light. ■

Alison Abbott is Nature's senior European correspondent.

Seriously out of synch

Imagine the impact on your working and social life if you regularly woke at 3 a.m. and had to retire to bed by early evening. That is the burden of people with a rare inherited condition called familial advanced sleep phase syndrome (FASPS). Their problems lie with a circadian clock that is out of synch with the daily cycle of light and dark¹⁰.

Circadian biologists are now looking at genes that are known to influence our body clock, to see if they can explain such extreme sleep disorders. In one FASPS family, the cause turned out to be a mutation in the human *Period2* gene¹¹. "It doesn't explain all cases of FASPS because this mutation was found in only one of the 40 families we are looking at," says Louis Ptáček of the University of California, San Francisco, who led the study. His group is now sequencing all of the known clock genes in each of the families in the hope of finding other mutations related to the syndrome.

Children with the rare but very debilitating Smith-Magenis syndrome, caused by a deletion in chromosome 17 that results in severe mental retardation, also have unusual circadian rhythms. Their production of the clock-setting hormone melatonin during the 24-hour cycle is essentially

reversed, so they do not sleep at night and become hyperactive while trying to stay awake during the day.

Earlier this year, clinical geneticist Hélène de Leersnyder at the Necker Hospital for Sick Children in Paris showed that these children can be treated by giving them beta-blockers in the morning to block melatonin secretion, and then giving them melatonin itself in the evening¹². "The children sleep more normally and their behaviour becomes more stable," she says. She is also looking for the deleted gene that may be responsible for their faulty clock.

Researchers led by Takashi Ebisawa, a neurogeneticist at Saitama Medical School in Japan, have found an association between the human *Period3* gene and a disorder called delayed sleep phase syndrome (DSPS), in which those afflicted typically stay up until 4 or 5 a.m. Intriguingly, Malcolm von Schantz and his colleagues at the University of Surrey in Guildford, UK, have also found that normal preferences for 'morningness' and 'eveningness' are associated with variations in the same gene¹³. Perhaps distressing conditions such as DSPS are just the extreme end of natural variation in the way our body clocks are set.

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Middle East: trying to break down the barriers

Collaborations are welcome, but can science ever thrive while the conflict continues?

Sir — Your News Feature “Across the great divide” (*Nature* **425**, 444–449; 2003) left me feeling despondent. As a Palestinian cell biologist, I am in favour of collaborations between open-minded Palestinian and Israeli scientists. This is for the benefit of both parties and will no doubt help in bridging the gap between the two nations. However, unless there is a huge investment in the Palestinian higher education system, this collaboration will continue to be on an unequal footing.

Israel has been illegally occupying and building settlements on Palestinian land since 1967. For many years, Israel has been subjecting the Palestinians to humiliation and collective punishment, including closing their schools and higher education institutions for long periods of time. This is done in the name of security — although during the past three years, while Palestinian militants have killed some 800 Israelis, the Israeli army has killed more than 2,200 Palestinians.

A disturbing fact is that the vast majority of casualties on both sides are innocent civilians, including many children. In my opinion, the only way forward is to end the Israeli occupation of Palestinian land and relieve the daily suffering of ordinary Palestinians.

I am one of hundreds, perhaps thousands, of Palestinian scientists who are working abroad, waiting for the opportunity to go back to Palestine and serve science and education in our own

homeland. We had a glimmer of hope with the Oslo agreements in 1996, and many Palestinian scientists returned at that time, but their dreams and lives were soon shattered by the current crisis.

A major obstacle to Palestinian scientists wishing to return home is the lack of infrastructure and funds for research at Palestinian institutions. Perhaps we should create more opportunities for Israeli scientists in Israel to collaborate with Palestinian scientists working in Europe or America, and wishing to return to Palestine in due course. This approach would be complementary to the existing local collaborations between Palestinian and Israeli institutions.

Bassam R. Ali

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Middle East: a picture is worth a thousand words

Sir — *Nature* has been careful to avoid taking a political stance in the Israeli–Palestinian conflict, and has taken a welcome position against the boycotting of Israeli scientists.

In this context, your News Feature “Across the great divide” (*Nature* **425**,

444–449; 2003) was mostly a well-balanced presentation of a complex situation, but I found the choice of graphics quite perturbing. The photographs used to illustrate the News Feature included two of Israeli military activity and three of the security wall, one of which appeared on the front cover.

Although these photos were provided by news agencies, I question the selection. It might have been more realistic, for example, if the youth seen scaling the wall in the cover photograph had been armed. There was only a single photo of the Israeli side of the conflict — a bus that had been destroyed by a suicide bomber — and one showing Hamas supporters.

The article correctly points out how personal this conflict is. I myself have been at a number of funerals and condolence calls for victims of suicide attacks, and have narrowly escaped being a victim. Despite this, I still hope for a peaceful solution to the conflict.

I feel that despite its shortcomings, and its negative appearance in photographs, the security barrier remains the least violent way currently available to improve the security situation here.

A picture is worth a thousand words — and *Nature* should be as careful in its choice of graphics as it is in its choice of words.

Joel Bigman

*Address withheld by request
Haifa, Israel*

Timaeus's insight on the shape of the Universe

Sir — With reference to the Letter to *Nature* “Dodecahedral space topology as an explanation for weak wide-angle temperature correlations in the cosmic microwave background” by J. P. Luminet *et al.* (*Nature* **425**, 593–595; 2003), perhaps it is worth recalling that a similar intuition struck Timaeus of Locri 2,500 years ago.

Timaeus (fifth century BC) was a Pythagorean philosopher from Locri, in Magna Graecia, now southern Italy. He was also a mathematician and astronomer. Plato studied with him while he was visiting Magna Graecia, and later wrote a dialogue presenting Timaeus in discussion with Socrates.

Out of the five regular, or Platonic, solids (tetrahedron, octahedron, cube, icosahedron and dodecahedron), Plato

tells how Timaeus thought of a mystical correspondence between the first four and the four natural ‘elements’ (fire, air, earth and water). As for the dodecahedron, the fifth regular solid, Timaeus regarded it as a shape that envelopes the whole Universe.

Claudio Giomini

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Chemists enthusiastic to embrace change

Sir — Your coverage of the recent American Chemical Society meeting in New York (“Chemists seek image overhaul,” *Nature* **425**, 227; 2003) described a symposium that was barely recognizable to those participating.

Far from involving an “image overhaul”, the discussion centred on reinventing

chemistry education so that it better fits the science of today.

The goal is to improve the training of scientists who will be working in an increasingly interdisciplinary environment.

By quoting comments that were made on the periphery of the discussion, your News story did not convey the enthusiasm expressed by many members for embracing change.

Indeed, the symposium — and the major conference that preceded it — are merely the first steps in what will, we feel, be a sea change in science education that will involve all our members.

Daryle Busch, Eli Pearce

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Contributions to Correspondence may be submitted to corres@nature.com. They should be no longer than 500 words, and ideally shorter. Published contributions are edited.

Completing the helix trilogy

Maurice Wilkins, who shared a Nobel with Crick and Watson, tells his story.

The Third Man of the Double Helix: The Autobiography of Maurice Wilkins

by Maurice Wilkins

Oxford University Press: 2003. 274 pp.

£16.99, \$27.50

Raymond Gosling

The first story of the discovery of the structure of DNA was *The Double Helix*, Jim Watson's racy account in 1968. Twenty years later, Francis Crick published *What Mad Pursuit*, a more scholarly heavy-going personal view of scientific discovery. The Nobel laureates' trilogy is now completed by Maurice Wilkins' autobiography, *The Third Man of the Double Helix*. In addition, we have Brenda Maddox's very readable biography of Rosalind Franklin (see *Nature* **418**, 725–726; 2002). What shines through in all of these books is their common agreement that it is the importance of DNA that makes the story of the people concerned worth telling. As Crick said: "It is the molecule that has the glamour, not the scientists."

Wilkins' autobiography gives us an intimate picture of a thoughtful, introspective man who has devoted his life to the pursuit of science and its impact on society. The buoyant sense of fun in the labs at King's College London, under the strong direction of John Randall, does not emerge as clearly as it might. For the lay reader I doubt if things will leap off the page as they do in Watson's *The Double Helix*.

In the first three chapters, Wilkins sets out his ancestry. He establishes the provenance of his social conscience, which has so influenced his work on the social impact of science over the past two decades. But for me the book only really comes alive when we reach chapter four, "Randall's circus". However, this may be because I joined the 'circus' in 1949 and can subconsciously fill in the backdrop to the words from my first-hand knowledge. Although I didn't know until I read the book that Wilkins had tried, unsuccessfully, to persuade Randall to give Crick a job at King's College — ah, if only!

The next chapter takes the reader into the detail of DNA. This is set well into a general context, showing the breadth of Wilkins' interests and contacts with other scientists. However, his account of his first meeting with Franklin seems to be coloured heavily with hindsight. This chapter gets to the heart of their relationship, describing the growing polarization between them in their different approaches to seeking the structure of DNA. It is worth getting this book just for this chapter and the next, which portray the way that successful scientific research is often actually done — a crab-like procession of mistakes and



Maurice Wilkins (left) studied nucleic acids at King's College London under John Randall (right).

muddle, leading ultimately to the truth. Not at all the logical process set out in the published papers, which were written after the event.

It is fascinating to learn how close the labs in Cambridge and London came to arranging formal collaboration on DNA research, but that Wilkins turned it down, apparently with Randall's support. This led to a moratorium agreed by Randall and Lawrence Bragg, director of the Cavendish Laboratory in Cambridge, that Watson and Crick should stop working on DNA modelling.

In the chapter entitled "The Double Helix", Wilkins lays claim to having realized that Erwin Chargaff's base ratios were important to the structure of DNA. But he admits that he had not realized that specific pairing linking the phosphate-sugar strands to one another with equivalent X-ray scattering ties would explain the predominantly helical diffraction pattern, which would be unaltered by any combination of base sequences. This has always seemed to me to have been the quantum leap that Watson made, and shows the advantages of model-building.

Wilkins eloquently describes his feelings at seeing the double-helix structure for the first time: "It seemed that non-living atoms and chemical bonds had come together to form life itself. I was rather stunned by it all." This sums up beautifully how Franklin and I felt. It was so elegant an explanation of all of the complex properties required of DNA, and contained so many elements familiar from our own work using X-ray diffraction. At the time I did not know that Wilkins was offered co-authorship by Watson and Crick, but refused. It would certainly have been appropriate, and seems to be something that he later came to regret.

In discussing the model, Wilkins mentions many of the scientists concerned with laying the foundation of knowledge on which the structure rests. He perceptively writes:

"as science becomes increasingly global and interdisciplinary, the idea of anyone standing up like someone at the top of Everest becomes increasingly inappropriate." He goes on to discuss the work that he, Herbert Wilson and their colleagues at Kings did over the next seven years to establish the correctness of the Watson-Crick model. He also writes charmingly that it was during this period that he met Patricia, who has mellowed this lonely, doubt-torn man into a happy father of four.

Chapters four to nine make most informative and interesting reading, from the discussions of X-ray diffraction to how it feels to be told by a journalist you've never heard of that you have won the Nobel Prize. However, there are three omissions from the account of the period surrounding the 1960 Lasker award, won by Wilkins, Crick and Watson: Wilkins' election to the Royal Society in 1959, his being made Companion of the British Empire in 1962, and the knighthood given to Randall for services to science in 1962. So many personal details are proffered to the reader, yet these events are excluded; I wondered why.

I have mixed feelings about this book, written as it is by someone I worked closely with and greatly admire. I sometimes found his 'warts and all' approach to the details of his life almost embarrassing, and feel a little resentful that I will now share such intimacy with the thousands who will read the book. However, this personal account of the way in which important research was conducted may well achieve one of the author's intentions, making everyone realize that they can, and should, make the effort to play a part in influencing the way that science is shaping our world. ■

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Hearty fare?

Coronary Artery Disease: Genes, Drugs, and the Agricultural Connection

by Ole Færgeman

Elsevier: 2003. 152 pp. 050, \$50

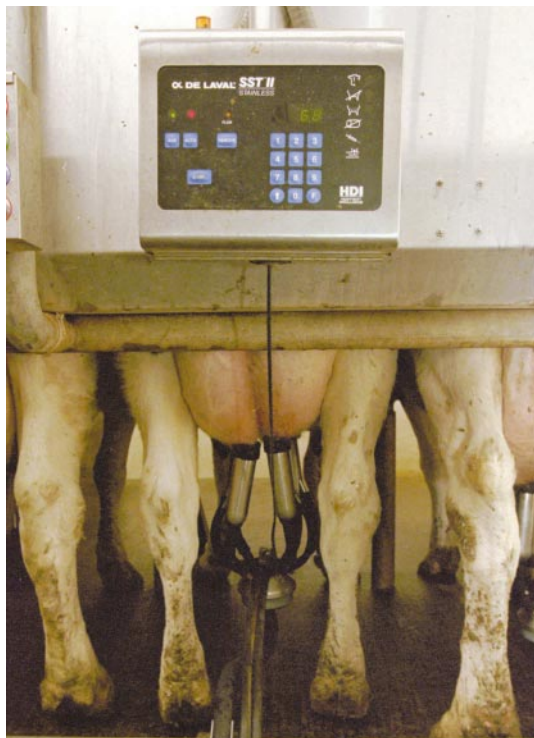
Marion Nestle

We can prevent coronary artery disease (CAD), says Ole Færgeman, professor of preventive cardiology at the University of Aarhus in Denmark. All we have to do is stop smoking cigarettes and stop eating the meat and milk of large domesticated land mammals. As Færgeman argues in this intriguing, provocative and sometimes maddening book, “producing huge numbers of animals for human food is at the core of the problems of agriculture, medicine, and global food security”. If we fail to grasp the evils of animal agriculture, it is because “the love of milk has been etched into our genome”, the “medico-industrial complex” promotes disease treatment rather than prevention, and powerful agricultural interests profit from our love of meat and dairy foods.

Never mind that agriculture brought us stability and abundant calories. It also brought vitamin and mineral deficiencies (due to a lack of food diversity), infectious diseases (acquired from animals), and poverty (from dependence and low wages). Agriculture is “the most environmentally destructive of all human activities”, wasting nitrogen — which causes “hypoxia in the sea as well as in the human heart muscle” — eroding soil, and is so inefficient that only 10% of the energy in fertilizer, tillage and transport is converted to food. “A doctor’s prescription for a better environment would be to lower the number of livestock... As a side-effect, it would reduce heart disease.”

Before dismissing such contentions as absurdly exaggerated, ponder Færgeman’s initial questions. When did CAD become an important cause of mortality, and why? How well do we understand its response to dietary factors? Why do we prefer to fund research on the genetics of CAD rather than its behavioural determinants? How does evidence-based medicine affect our understanding of the social, political and agricultural causes of CAD? And how can we answer such questions when research is so often sponsored by self-interested industries?

Færgeman brings plenty of clinical experience to bear on these issues. He has witnessed the effects of saturated animal fat on blood cholesterol levels, of blood cholesterol on plaque formation, and of plaque rupture on coronary thrombosis. Epidemiological studies “clinch the arguments”: populations consuming food from land mammals have



Milk bar? Ole Færgeman believes that reducing our intake of dairy products will prevent coronary artery disease.

higher rates of CAD but live longer when they replace beef and butter with fruits, vegetables, nuts and fish. Plant-based diets not only lower blood cholesterol but also protect against arterial inflammation, endothelial malfunction and disturbances of heart rhythm. Drugs help, but they address symptoms, not causes. Genetics matters, but not much: possessing the wrong genes rarely causes CAD unless people also eat meat and drink milk.

I am guessing that Færgeman is not a vegan, but such statements make him sound like one. The book’s startling cover image, derived from Nordic myth, depicts a rather well-endowed man suckling milk from a cow. Adults, he says, are not supposed to drink milk. Evolution ensures that infants wean; most people lose the ability to digest lactose (milk sugar) after early childhood. The rare persistence of lactase in certain populations is “perhaps the most important single genetic determinant of risk of coronary artery disease”. If people fight evolution by continuing to drink milk, it is because the medico-industrial complex labels lactase “deficiency” as a genetic disorder and the dairy industry has convinced us that its products are essential.

I am at a loss to decide whether Færgeman would make a lively or tedious dinner companion. He writes well, but digresses. Along with matters clearly germane to CAD, he discusses theories of biological complexity, Danish history, the career of Tycho Brahe, Prozac, psychological debriefings, gallstone

surgery, anaesthesiology and the fractal coastline of California. Such digressions slow the pace and detract from the core arguments, which are much more fun. He rails against the politics of medicine, the influence of corporations on universities and the limitations of science in answering questions about behaviour and health. He worries about how science is corrupted by the meat, dairy, tobacco and pharmaceutical industries and how evidence-based medicine is inadequate to deal with the environmental causes of disease. He agrees that clinical trials make good science but regrets that they leave few resources for studying the political, societal and agricultural context of disease.

Alas, Færgeman offers few solutions to these problems. Unless we change our ways, he warns, the unwise will continue to succumb to CAD, and agriculture will continue to determine the need for cardiologists and heart surgeons. Govern-

ments should integrate health and agricultural policies, researchers should study social determinants of disease, and universities should remain independent of corporate influence. It’s up to us, he says, to “develop the political will to ... protect nature and to promote human health and sanity”. Yes, but how? This book is about policy, not dietary practices or political advocacy, and Færgeman leaves us on our own to work out how to apply his important lessons. ■

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Dear diary...

Science, Not Art: Ten Scientists’ Diaries

edited by Jon Turney

Calouste Gulbenkian Foundation: 2003. 160 pp. £8.50

Nancy Rothwell,
with Siobhan Blagbrough

The title of this book is quite misleading. *Science, Not Art* conjures up visions of a contribution to the topical debate on the similarities, differences and distinctions between science and the arts. But it’s not about that at all, as clearly stated in the preface. Its title arises from an earlier popular book, *Art, Not Chance*, which was produced by the same



Under pressure: space physiologist Kevin Fong feels the strain of combining studies of astronauts' health with life as a junior doctor at a London hospital.

publisher and comprised extracts from the diaries of nine leading artists.

Science, Not Art follows the same pattern, with short passages from the diaries of ten scientists, accompanied by excellent photographs by Hugo Glendinning. The individuals may not yet be world leaders in their fields, but they are certainly not "ordinary young scientists". Many hold prestigious Royal Society University Research Fellowships in disciplines encompassing mathematics, cosmology, biology, chemistry and medicine. The scientists also challenge the title of the book, as many have strong links with art, music and popular science writing. Two hold fellowships from the National Endowment for Science, Technology and the Arts, which are awarded to creative individuals, particularly for work spanning science and arts. Several have formal links with the arts and artists.

The diaries are well written and engaging, and are all very different in style. Some of the scientists focus mainly on their research, whereas others play heavily on their personal lives and the tensions between a heavy workload and family life. Some are in note form; others tell fascinating stories. Particularly engaging are the tales of Jon Copley, a marine biologist encountering a storm on a scientific voyage, and the experiences of Kevin Fong, a young clinical researcher, in a busy intensive-care unit in a London hospital.

Most remarkable are the common features of the diaries. The scientists all write of pressure, devotion and great commitment, the depressing lows and great highs accompanying failure and success in their scientific lives — and these often seem to have greater impact than personal events. They all tell of the importance of working with people, of the excitement of discovery, and of the

despair that follows unsuccessful grant applications, substandard scientific presentations and poor reviewers' comments on submitted manuscripts.

The audience for this book is not obvious. Scientists will enjoy the comparisons and familiarity of many of the stories, and feel relief at the shared problems and concerns, but the nature of the book and its clear, jargon-free explanation of science should attract non-scientists. To test this, I asked a 16-year-old, Siobhan Blagbrough, who has no current aspiration to enter science, for her opinion.

"I found the book interesting and surprisingly easy to read. While dealing with scientific concepts beyond my understanding, the personal approach and diary format meant that there was usually something to keep me interested. The way that the different scientists became involved in their field, sometimes without following the expected academic route, made me think that their success stems more from their determination to find solutions to questions that on the face of it may appear trivial or at least of little apparent value. But they can see how these fit into a bigger picture and may lead to a major breakthrough.

"To read how they cope with daily life and problems but still have the determination to overcome this and work so hard on their various projects is quite inspiring. I also found exciting the way that many of them travel all over the world, meeting other scientists to learn from each other.

"I would say that the book gave me an insight into these scientists' lives and how, in many aspects, they are similar to everyone else, fearing failure or rejection but perhaps having more dedication, determination and vision than most."

This summary is perhaps a better recommendation than I could make.

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Quantity control

Measure for Measure: The Story of Imperial, Metric, and Other Units

by Alex Hebra

Johns Hopkins University Press: 2003.

232 pp. \$24.95, £18.50

David Lindley

In the United States people take their temperatures in Fahrenheit and, more mysteriously, rate their air-conditioners in British thermal units (Btus). Experience shows that it takes a few thousand Btus to cool a typical room (volume measured in cubic feet, of course, and let me not get started on the fact that we build our houses using two-by-fours whose cross-section is an inch and a half by three-and-a-half inches). Scientists may find this lamentable, but in everyday use scientific units have no particular advantage. Working in approved units, you would have to buy an air-conditioner with a capacity (pause while reviewer uses Google to find the definition of Btu) of some number of megajoules. To the average shopper, this is equally incomprehensible.

In this haphazard and only fitfully engaging book, Alex Hebra conveys clearly enough that convenience and tradition account for the origin of most of the units we use, as well as the fact that even scientists haven't completely fallen in line with the *Système Internationale*. Cosmologists insist

on their megaparsecs, and high-energy physicists remain attached to the electron-volt. You don't hear much talk of gigapascals on the evening weather forecast.

Hebra devotes a chapter each to time, length, mass, heat and so on, and offers some entertaining tidbits about the genesis of what we are now pleased to call standard units. It is hard to know what sort of reader he is addressing. He explains at some length that the second is one-sixtieth of one-sixtieth of one twenty-fourth of a solar day, because the Babylonians were fond of twelves (although they forgot to tell us why), and he takes a couple of muddled pages to sketch the oddities of Earth's orbit that make the solar day an elusive and untrustworthy quantity. But then he finishes by saying in a single brief paragraph that an official second is now so many vibrations of a caesium atom. He doesn't think it necessary to explain what sort of vibrations he means or how they are counted with the necessary accuracy.

Although Hebra is described as a science writer and engineering consultant, scientific explanation is not his strength. In the chapter on heat and energy, he confuses phlogiston with caloric, claims that James Watt invented the steam engine and thereby proved that phlogiston does not exist, and says that Kelvin established the absolute zero of temperature by observing that the ideal gas law implies zero volume at a sufficient degree of cold. In fact, this point had been a century and half earlier, but its significance was far from clear. Kelvin instead used Carnot's theory of heat engines to define a temperature scale through mechanics, and only after taking hints from Joule and Clausius did he realize there must be an absolute zero.

Hebra's obvious preference for engineering over science produces some of the better parts of the book. I was pleased to learn that viscosity is sometimes measured in Engler's degrees, which are related, naturally enough, to the speed with which a fluid passes through an Engler viscometer. If you're trying to figure out what diameter of piping to use in your molasses factory, this may well be a handy number. Quick: what's viscosity in SI units?

The author finishes with a modest plea that the United States might one day summon the nerve to go wholeheartedly metric. But he admits that this would cost a huge amount of money, and in any case many of his examples demonstrate the possibly unintended point that practicality often wins out over scientific rationality for perfectly good reasons — or at least reasonably good reasons. Perhaps manufacturers of electrical appliances should offer to give up British thermal units provided that astrophysicists agree to banish parsecs and solar masses. ■

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Science in culture



Green revolution: Wilhelm Ostwald's paintings put his theories about colour into practice.

Painting by numbers

Chemist Wilhelm Ostwald added colour to the art world.

Philip Ball

This picture might look like the barely competent effort of a disciple of Caspar David Friedrich, but Wilhelm Ostwald intended that his deeply romantic seascape would, by virtue of its 'scientific' colouring, be beyond criticism.

Ostwald, the 1909 Nobel laureate who pioneered the discipline of physical chemistry, was an enthusiastic amateur painter. His works, some of which have recently been published for the first time in *De Artes Chemiae* (Lothar Beyer and Rainer Behrends, Passage, 2003), show that he was not without talent, although he was stylistically conservative and was more or less untouched by modernism.

But Ostwald's artistic endeavours are more than a marginal curiosity, for his theories on colour were hugely influential in the early decades of the twentieth century, in both fine art and industry. Ostwald's paintings reveal how he thought his ideas should be put into practice.

There is a long-standing view that red, yellow and blue are the only three primary colours, but Ostwald defied this: in his *Colour Primer* (1916), he included green as a primary, later awarding it a large segment of his colour wheel. While not of course denying that green can be mixed from blue and yellow, he followed the psychologist Ewald Hering in asserting that green is perceptually autonomous. Ostwald's colour theory was much discussed by Piet Mondrian and his colleagues in the De Stijl group of painters, and may have led Mondrian to use a distinctly greenish yellow in his grid-like compositions of around 1920, as though he was trying to accommodate yellow and green in a single primary.

The most important aspect of Ostwald's colour theory, however, was the role he assigned to grey. His attempts to map colour space followed those of Albert Munsell, whom Ostwald met in 1905. Munsell tried to quantify and standardize colours according to parameters of hue, saturation and brightness. The

last of these was particularly important to Ostwald, who introduced a grey scale into colour space. He believed that a scale of perceptually equal steps in the brightness of a colour could be achieved by adding black and white in ratios that followed a logarithmic progression. This, he said, provided a scheme for achieving perfect tonal balance and harmonious colour composition in a painting.

Ostwald used his fame as a chemist to impress his colour theory on the German paint industry. In 1912 he joined the Deutsche Werkbund, an organization dedicated to introducing standardization into industrial design, and in 1914 he arranged an exhibition of commercial paints and dyes. Eventually Ostwald established his own pigment factory, called Energie.

The idea that colour composition could be pursued in an objective, 'scientific' way found echoes in the 1920s at the Bauhaus school of art and design in Weimar, Germany, where Paul Klee and Wassily Kandinsky taught. Kandinsky believed that a carefully chosen arrangement of colours could pluck the emotional strings of the soul as deliberately as a pianist strikes notes on the keyboard. He assigned dogmatic meanings, established through psychological tests, to specific colours.

Ostwald was a controversial figure at the Bauhaus, where he lectured in 1927. He was asked to join the advisory board the following year, but his response is not recorded. His eagerness to 'correct' painters who did not use colour 'properly' — he once announced that Titian had used a blue two tones too high — grated on the sensibilities of some artists. Kandinsky became sympathetic to Ostwald's ideas, but Klee was unwilling to be fettered by any scientific theory of colour. Ostwald's rules, he said, meant "renouncing the wealth of the soul. Thanks, but no thanks."

♦ www.wilhelm-ostwald.de

Philip Ball is a consultant editor of *Nature* and author of *Bright Earth: The Invention of Colour* (Viking, 2001).

Written in blood

Lance A. Liotta, Mauro Ferrari,
Emanuel Petricoin

The blood contains a treasure trove of previously unstudied biomarkers that could reflect the ongoing physiologic state of all tissues. Every cell in the body leaves a record of its physiological state in the products it sheds to the blood, either as waste or as signals to neighbouring cells. What some may view as a cellular refuse is really a diagnostic gold mine.

Routine laboratory blood tests sample only a minute fraction of this potential repository, and there are few specific markers for life-threatening diseases such as cancer. The current biomarker repertoire cannot detect treatable early-stage cancer and often misclassifies common benign conditions. In the face of the urgent need for better disease markers, it is unfortunate that the number of new markers submitted for regulatory approval has virtually dried up.

It is time to rethink our approach to biomarker discovery. The quest for a single biomarker for a particular disease has the illusion of analytical simplicity, but makes little sense from a biological perspective. For example, cancer is caused by intrinsically deranged cells of the host — not an external infectious agent. Why should we expect the cancer cell to generate a unique new protein? It is not surprising that we have failed to find an accurate single marker for a disease as heterogeneous as cancer, which comes in hundreds of types and stages and is a product of the tumour–host microenvironment. Instead, why not take advantage of the very complexity of the disease? Genomics researchers have moved beyond one-gene-at-a-time analysis, and are profiling thousands of gene transcripts to generate entire patterns of information. We should be doing the same with protein biomarkers.

The relative cellular abundance of tens of thousands of different proteins, along with their cleaved or modified forms, is a reflection of ongoing physiological and pathological events. For example, cells that succumb to programmed cell death will leave behind a different protein signature from cells dying of oxygen starvation or infectious insult. As tissues are perfused by blood and lymph, proteins and protein fragments passively or actively enter the circulation. Thus, the complex chemistry of the tumour–host microenvironment should generate unique signatures in the blood macroenvironment.

The serum proteome is a complex mixture predominated by high-abundance resident proteins, such as albumin and other carrier proteins, together with proteins

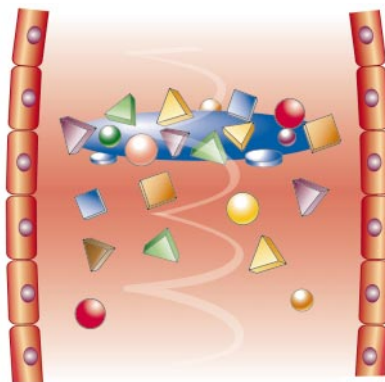
that originate from circulating blood cells. Although proteins entering the blood from the surrounding tissue are much less abundant, it is this fraction that is likely to contain most of the undiscovered biomarkers.

Large proteins only enter the bloodstream intact if they are actively secreted, or if the vascular wall becomes permeable owing to disease. But degradation and cleavage can generate fragments small enough to enter the blood passively, producing diagnostic traces. Thus the low-molecular-weight (LMW) region of the blood proteome, which is a mixture of small intact proteins plus fragments of the large proteins, represents all classes of proteins. This treasure trove of diagnostic information has largely been ignored until now.

The size of a protein determines how fast it is cleared from the blood by kidney filtration and uptake by the liver. Small proteins are rapidly cleared, with half-lives of less than a few hours, whereas large proteins have extended half-lives — albumin's, for example, is 19 days. Consequently, the only way a small molecule can stay in circulation is to hitch a ride with a carrier protein. Thus, at any point in time the concentration of a LMW biomarker is a function of the kinetics of its entry and exit, as well as its binding affinity for carrier proteins.

The carrier proteins act as magnets to accumulate, and thereby amplify, low-abundance biomarkers. A trickle of biomarkers entering the blood is mopped up by the carrier proteins, which amplify and integrate the history of biomarker production, as a capacitor stores electricity. Existing fractionation methodologies often discard abundant carrier proteins and thus fail to capture this valuable resource — akin to throwing the baby out with the bathwater.

Recognizing the existence of such amplification and enrichment should shift our future discovery efforts toward the constellation of carrier-protein-bound LMW biomarkers. Fortunately, mass spectrometry is a technique ideally suited for detecting and



Clinical proteomics

The low-molecular-weight region of the blood proteome is a treasure trove of diagnostic information ready to be harvested by nanotechnology.

distinguishing thousands of LMW proteins and peptides in seconds. In the near future, we will be able to scan the blood proteome rapidly by mass spectrometry, decipher the buried diagnostic information, and then go directly to a list of the underlying identities in a database. Artificial-intelligence-type algorithms can sort through the information contained in thousands of data points, recognizing and exposing disease portraits.

We can envision a future in which the blood-biomarker archive can be monitored with new technology created at the intersection of the fields of artificial intelligence, nanotechnology and proteomics. Advances in microfabrication may provide 'nanoharvesting' agents designed specifically to capture and amplify classes of LMW biomarkers. Imagine a fleet of harvesting agents tailored to monitor specific diseases. Harvesting particles could be administered in the physician's office, and then sampled at a later visit after they have had time to collect their diagnostic cargo. A serum sample containing LMW diagnostic molecules sequestered on the harvesters can now be rapidly analysed with mass spectrometry. The result could be an individual global-health profile rendered at an affordable cost, revolutionizing the field of disease diagnosis and health monitoring. Perhaps there will be a time in which a small sample of blood will reveal an image of the physiological and pathological states of every tissue in the body. ■

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BAC-to-BAC images of the brain

Huda Y. Zoghbi

A large-scale effort to uncover the gene-expression profiles of individual neurons and create a demographic atlas of the brain is under way. First data from this project are revealing new information about neuronal development.

For centuries, avenues to understanding the nervous system were confined to post-mortem anatomical studies and clinical portraits of the brain in disease states. The first approach has produced a fairly good map of the brain; the second, a catalogue of behaviours that we can trace with a reasonable amount of accuracy to distinct brain regions. The challenge of neurobiology has been to bridge the gap between map and catalogue — to understand how the brain's billions of neurons and their myriad connections mediate perception, behaviour, learning, memory and consciousness. In a large-scale effort spanning several institutions, Gong and colleagues¹ have now taken a big step in this direction. They have used genetically engineered mice and the tools of gene-expression analysis to begin constructing what is akin to a demographic atlas of the brain, identifying patterns of gene expression in individual neurons, and revealing differences between cells that were hitherto thought to be identical. They present the first data from this project on page 917 of this issue.

By identifying different patterns of gene expression, we can classify the various cell types in the central nervous system by their molecular characteristics and function, rather than by their appearance. For example, all Purkinje cells — the neurons that control coordination and movement — look identical, but some make the protein zebrin, whereas others do not². Although we do not yet understand the significance of such differences, it is likely that many neurons that look similar have different gene-expression profiles, and therefore different functions. But information about gene expression in individual neurons has been exceedingly difficult to come by.

Technical approaches to obtaining this information are immunohistochemistry and RNA *in situ* hybridization. In immunohistochemistry, an antibody to a specific protein is used to identify and mark cells that are producing the protein, whereas in RNA *in situ* hybridization, neurons expressing particular genes are labelled using nucleic-acid probes that bind to specific messenger RNA (mRNA) sequences inside cells. Unfortunately, RNA *in situ* hybridization does not provide detailed images of neuronal projections, information that is vital to understanding how populations of neurons

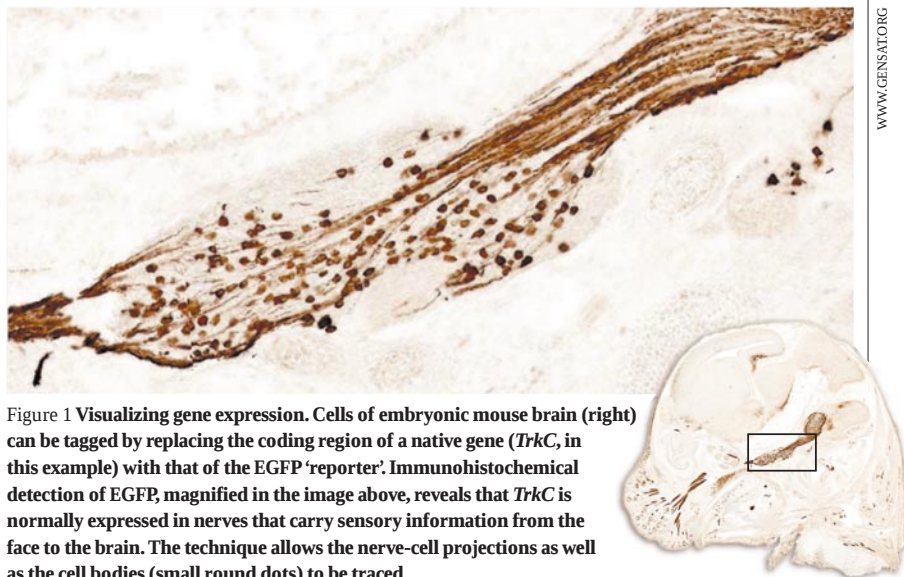


Figure 1 Visualizing gene expression. Cells of embryonic mouse brain (right) can be tagged by replacing the coding region of a native gene (*TrkC*, in this example) with that of the EGFP 'reporter'. Immunohistochemical detection of EGFP, magnified in the image above, reveals that *TrkC* is normally expressed in nerves that carry sensory information from the face to the brain. The technique allows the nerve-cell projections as well as the cell bodies (small round dots) to be traced.

interact. What is needed to create a function-based map of the brain is a practical way to visualize the interconnections between neurons and to approach neuronal function *in vivo*, without chemical treatment.

In attempting to fill this need, Gong *et al.*¹ have capitalized on another large-scale map — the genome sequence of the mouse. Most of the mouse genome is cloned as large fragments (150–200 kilobases in size) in so-called bacterial artificial chromosomes (BACs) — vectors that can be easily propagated inside cells. The advantage of BACs is that they are usually large enough to carry both the coding region of a gene and its regulatory sequences, which determine where and when the gene is expressed. Using this library of BACs, Gong and collaborators have begun to systematically replace the coding regions of different genes with a 'reporter' sequence that encodes a fluorescent protein (enhanced green fluorescent protein, or EGFP)³. Injecting each modified BAC into a mouse egg, and generating transgenic animals, enables Gong and colleagues to trace when and where each gene is expressed in the central nervous system — the patterns of EGFP production reflect the natural expression patterns of each gene (Fig. 1). The project, called the Gene Expression Nervous System Atlas (GENSAT) BAC Transgenic Project, has already provided data on some 150 genes, but it

aims to provide detailed expression maps for thousands more.

Using conventional mRNA *in situ* hybridization or immunohistochemistry, Gong *et al.* have attempted to corroborate the data they have obtained from the transgenic mice. They have found that authentic EGFP expression patterns are produced if sufficient regulatory sequences are included in the BAC. Unfortunately, this method does not work well with very large genes (those around 250 kilobases or more in size), as they do not fit on a single BAC with all the requisite regulatory sequences. About 10% of the transgenic-mouse lines produced aberrant expression patterns as a result of this limitation.

The authors also noticed that several copies of certain BACs were required in each mouse cell in order to visualize EGFP — the signal was not strong enough from a single copy. This is a reminder that the EGFP RNA or protein might be less stable than the products of the replaced gene. In this regard, the BAC strategy has an advantage over the EGFP 'knock-in' strategy, in which the mouse chromosomes (as opposed to cloned genetic fragments) are modified, so that EGFP replaces the endogenous coding region of a gene. Fluorescence from a single-copy EGFP knock-in gene can often escape detection.

The first 150 genes of the GENSAT project

have already produced several biological insights. One of the genes investigated is the 'goosecoid-like' gene (*Gsc*), which is missing in people with DiGeorge syndrome (a disorder characterized by congenital heart defects and learning difficulties, among other developmental abnormalities). Using BAC transgenic mice to trace the patterns of *Gsc* expression, Gong *et al.* found that the protein encoded by this gene is normally produced by neurons in a brain region that regulates rapid-eye-movement (REM) sleep. The authors also identified the two embryonic cells that give rise to this population of neurons. The knowledge that these cells express *Gsc* should alert investigators to subtle alterations in REM sleep in DiGeorge patients and in mouse models of the disorder⁴. In other instances, the EGFP approach allowed Gong *et al.* to trace neuronal projections and to identify the migration patterns of particular neurons during development. The project has also revealed specific cell classes within the different layers of the cerebral cortex — the outermost region of the brain responsible for functions such as sensory perception, thought and reasoning.

What future insights are the GENSAT data likely to provide? Many neurological disorders affect specific groups of neurons, but we need to know how the malfunctioning population affects other neurons. Parkinson's disease and Tourette's syndrome, for example, affect neurons in a brain region called the striatum. GENSAT data could, by distinguishing the projection patterns of different striatal neurons, facilitate studies of the physiology and cell biology of these neurological disorders. Even greater insight might come from identifying genetic differences between neurons of the same class. This could increase our understanding of brain disorders in which neurons look normal despite obvious behavioural dysfunction, as is the case with schizophrenia or autism.

In the short term, neuroscientists can learn where their favourite gene is expressed, which might inform their studies into the functional consequences of its loss. Cells expressing the EGFP reporter can be identified and purified by fluorescence-activated cell sorting, so that their gene-expression profiles can be examined in more detail. The EGFP reporter strategy could also be of clinical benefit if it were used to evaluate drug treatments for neurological diseases. Prospective therapies could be tested in BAC transgenic mice, in which the level of EGFP fluorescence would be a direct read-out of a drug's effects.

Meanwhile, the GENSAT database⁵, which is publicly available, will be updated as soon as new data are generated. As it matures, the wealth of information therein will, no doubt, prompt many new questions. Their answers will lead us further towards

understanding the topology and demographics of the brain.

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Black holes

Sparks of interest

Ramesh Narayan

Why is the black hole at the centre of our Galaxy so dim, when those in other galaxies can outshine the stars around them? Newly discovered bursts of infrared radiation may give the first clues to what is going on.

At the centre of the Milky Way is a supermassive black hole called Sagittarius A* (Sgr A*)^{1,2}. As supermassive black holes go, Sgr A* is a relatively small one: it's four million times more massive than the Sun, but black holes up to 1,000 times more massive are known to exist in other galaxies. What makes Sgr A* special is that it is by far the closest supermassive black hole to Earth, making it a prime target for study. And, of course, it is our black hole, at the centre of our own Galaxy. Another, curious, feature is that Sgr A* is one of the dimmest black holes known.

Sgr A* has been studied extensively at

long wavelengths, through the detection of radio- and millimetre-wavelength radiation from it; only recently has that information been complemented by images at shorter wavelengths, taken by the space-borne Chandra X-ray Observatory³. On page 934 of this issue, Genzel and colleagues⁴ add more detail, with their detection of Sgr A* at infrared wavelengths, using the Very Large Telescope in Chile's Atacama Desert. Genzel *et al.*, and another group led by Ghez⁵, find that the brightness of Sgr A* at infrared wavelengths is highly variable, and flares frequently. These observations, reflecting similar patterns seen earlier in X-rays, open

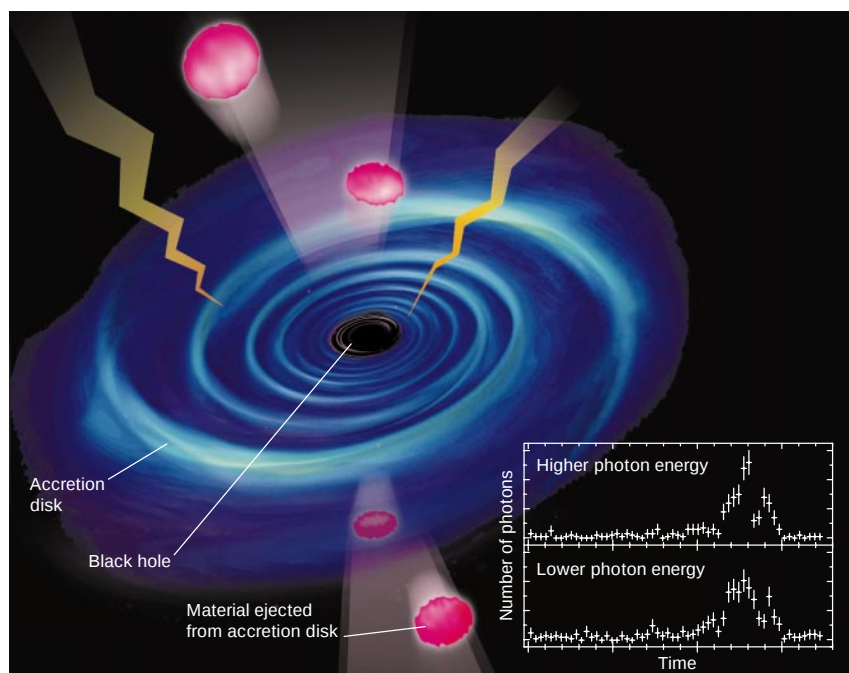


Figure 1 Unsteady accretion. Hot gas swirls in an accretion flow, as it is gradually sucked into the supermassive black hole at the centre of our Galaxy. The energy of the gas is converted into radiation, which is emitted in sudden bursts. Genzel *et al.*⁴ have detected several flares of radiation at infrared wavelengths, which may result from the emission of blobs of gas from the disk (accompanied by radiation) or from sparks that occur randomly in the accreting gas. The inset shows the earlier, similar detection of an X-ray flare³. Piecing together observations of the black hole across a range of wavelengths should reveal the complex physical processes that occur in accretion flows. (Based on an image created by Michael P. Owen.)

Physiology

Foreman in the bone factory

Etsuko Abe and colleagues have made an unexpected discovery, as they report in *Cell* (115, 151–162; 2003). They show that thyroid-stimulating hormone (TSH) — best known for its role in stimulating the secretion of thyroid hormones in mammals — also regulates the turnover of bone.

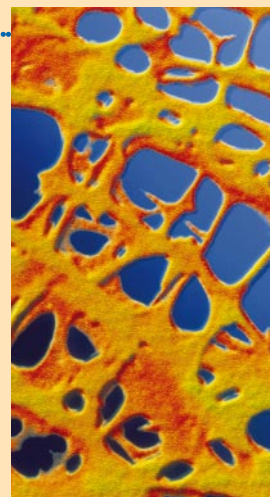
During adult life, old bone is continually resorbed and replaced by new bone. These 'remodelling' events must be tightly coupled in both space and time. If, for instance, the activity of bone-resorbing cells (called osteoclasts) exceeds that of bone-depositing cells (osteoblasts), the result is osteoporosis — brittle, weakened bones. The scanning electron micrograph opposite shows the spongy nature of such bones,

with many large holes visible (blue). A variety of factors can cause osteoporosis, one of which is high levels of thyroid hormone. Such high levels produce low TSH levels, but it has generally been thought that TSH has nothing to do with the reduction in bone density. Abe *et al.* now find otherwise.

The authors started by generating mice that lack the cellular receptor that responds to TSH. They found, not surprisingly, that the mice showed little thyroid activity and stunted growth (one of the thyroid's main functions is to ensure proper growth). But the animals also had short, overly light bones. The bones showed severe osteoporosis as well as localized dense patches, suggesting that bone formation and

resorption had, to some extent, become spatially separated. Feeding the mice thyroid extracts, to restore their levels of thyroid hormones, increased body weight, but not bone weight or length. This hints that the effects of TSH loss on bone are direct, rather than occurring via thyroid hormones. In support of this, the TSH receptor is found on both osteoclasts and osteoblasts.

Abe *et al.* also discovered that bone was remodelled much more quickly in the mutant mice than in normal animals, and that more osteoclasts and osteoblasts were formed. These and other findings suggest that TSH usually functions to inhibit both of these cell types. The authors have also begun to define the distinct intracellular signalling



pathways by which TSH prevents the formation and survival of osteoclasts and the formation of osteoblasts. The conclusion is that when TSH is missing, bone remodelling loses a crucial overseer. **Amanda Tromans**

a new window on this enigmatic source.

Some supermassive black holes in distant galaxies are observed as very bright quasi-stellar objects, or quasars, that often outshine an entire galaxy of stars. Those black holes accrete (absorb) a lot of gas from their surroundings and in so doing convert a good fraction, say 10%, of the mass energy of that gas to radiation. Their luminosities are nearly equal to the maximum allowed — the so-called Eddington limit, which is proportional to the mass of the object. Sgr A*, in contrast, is extremely dim, radiating at only a billionth of the Eddington limit for its mass. In this respect it resembles the vast majority of black holes in the Universe, which are mostly very dim.

Why is Sgr A* so dim? It is true that it has less gas to accrete, compared with the bright black holes in quasars — but this is only around 10,000 times less, not a billion times. Two other factors are believed to contribute to its dimness. First, in contrast to the accretion flows in quasars, the gas flow in Sgr A* is radiatively inefficient⁶: only a very small fraction of its mass energy is converted to radiation. Second, as a direct consequence of this radiative inefficiency, only a small fraction of the available gas actually accretes onto the black hole⁷, the rest being ejected from the system.

This still leaves the fundamental question of exactly how an accretion flow converts mass energy to radiation and why the process is very efficient in bright quasars but highly inefficient in Sgr A*. Something is clearly different about the physics in bright black holes and in dim ones. Unfortunately, the relevant effects are complex and poorly understood, and it has become clear in recent years that

real progress will be achieved only when we have more detailed observational clues. The infrared detections of Sgr A* by Genzel *et al.*⁴, coupled with Chandra's X-ray observations, may be the breakthrough we have been looking for.

The fact that the emission from Sgr A* varies over tens of minutes, and is almost periodic⁴, indicates that the radiation comes from gas orbiting close to the black hole. This is not unexpected. What is surprising is that Sgr A* emits frequent massive flares of radiation at both infrared and X-ray wavelengths, suggesting that the conversion of mass to radiation is not steady and continuous, but very erratic. There are several possible explanations. One is that the radiatively inefficient accretion flow ejects gas in spurts rather than continuously, and that each ejection of a blob of gas is accompanied by a spurt of radiation⁸. Another is that the amount of gas accreting onto the black hole itself fluctuates, causing the emission to vary⁹. A third idea, perhaps the simplest, is that the accretion engine shuts out once in a while. Lines of magnetic field pervade the accretion disk, and occasionally these may become so distorted that they 'snap' and new lines form. These magnetic reconnection events would produce streams of energetic particles and sparks of radiation¹⁰ (Fig. 1).

It is not clear at present which, if any, of these ideas is correct, or how the radiation processes actually work in detail. But the beauty of having a flaring source such as Sgr A* is that each flare provides a new and independent view of the underlying physical processes. So by collecting and studying data on many flares, we may learn much more than from a steady source.

The new discoveries about Sgr A* are the direct result of remarkable improvements in technology: the launch of Chandra in 1999, with its superb resolution for X-rays, and the commissioning of adaptive optics on both the Very Large Telescope and the W. M. Keck II Telescope in Hawaii, which allows exquisite observations at infrared wavelengths. A dedicated observational programme using these facilities, and other observatories for radio and sub-millimetre¹¹ wavelengths, is clearly the next step. From such observations we will discover whether flares occur simultaneously at all wavelengths (with different amplitudes), or whether the X-ray and infrared flares are largely independent of each other. If the flares are roughly simultaneous, what is the time delay between the emission in different bands? And how much, and in what direction, is the radiation polarized? The answers to these and related questions will doubtless reveal much about the physics of Sgr A* and other dim black holes. ■

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100 YEARS AGO

At the recent meeting of the British Association at Southport I heard numerous complaints (repetitions of those I have heard at not a few previous meetings) by the general public, members of the Association, on the too technical character of the papers read before it. These complaints referred to all the sections except, perhaps, those of anthropology, geography, and educational science. One overheard too often to be pleasant such remarks as "I am interested in zoology, but what is the good of coming to listen to such a paper as this? I have no idea what the speaker is talking about" — the paper, in one specific instance, was cytological, and of great value undoubtedly; and, "I have not gained much by becoming a member of the Association; the papers are all over my head." These complaints are being made by well educated men and women interested in science, but not versed in its technicalities... The general public have really some cause for complaint that their subscription has been obtained from them on a misunderstanding.

From *Nature* 29 October 1903.

50 YEARS AGO

The death of Dr. E. P. Hubble from a heart attack, on September 28 at the age of sixty-three, has robbed the world of astronomy of its leading authority on nebulae... On his return home in 1919 after the First World War, Hubble was appointed to the staff of the Mount Wilson Observatory. He soon established his position as a leading worker in the field of the nebulae. He showed that diffuse nebulae owe their illumination to stimulation by radiation from hot stars. He then turned to the classification of the spiral nebulae and showed conclusively that they are stellar systems lying outside our galaxy. He established a scale of distances from a study of the brightest stars and Cepheid variables in the nebulae. Working with Humason and the 100-in. telescope, he established the velocity-distance law from the red shifts of lines in the spectra; this increased the reliable distances to which astronomers could plumb the depths of space up to 250 million light-years. He studied the law of the red shifts in all its bearings, aiming always at cutting down the number of conflicting interpretations, and he kept always an open mind on the kinetic or other explanations of the red shifts.

From *Nature* 31 October 1953.

Palaeontology

Smart-winged pterosaurs

David M. Unwin

Why did ancient flying reptiles have so much processing-power in the back of their brain? To provide highly responsive flight control, is an answer to emerge from an innovative analysis of pterosaur skulls.

Brains, not surprisingly, are rarely fossilized, leaving a large gap in our knowledge of the anatomy of most extinct organisms. Fortunately, in some vertebrates — mammals, birds, dinosaurs and pterosaurs — the brain fits so tightly into the braincase that its external features are faithfully reflected by the contours of the inner surface of the bones that enclose it. Unfortunately, opportunities to recover these data from fossil material are infrequent and often involve destructive techniques, thereby excluding many valuable specimens from consideration.

High-resolution X-ray computed tomography, which has proved extremely helpful elsewhere in palaeontology¹, offers the possibility of looking inside braincases and generating a digital cast without damaging the fossil. Witmer and colleagues² have successfully applied this new technique to two kinds of pterosaur (see page 950 of this issue). These are a poorly understood group of flying reptiles that flourished during the Mesozoic (between 251 million and 65 million years ago) and which remain the subject of controversy³. The new work clarifies several aspects of pterosaur neural anatomy, and prompts some startling new ideas regarding their locomotion and behaviour.

Witmer *et al.* looked at rare, uncrushed skulls of two specimens. One was of *Rhamphorhynchus*, a long-tailed, crow-sized creature from the Upper Jurassic (163–144 million years ago). The other was of *Anhangura*, a large, short-tailed form dating to the Lower Cretaceous (144–97.5 million years ago). In trade jargon, *Rhamphorhynchus* and *Anhangura* are respectively 'basal' and 'derived' — loosely put, 'primitive' and 'advanced'.

The new findings confirm earlier studies^{4,5}

showing that pterosaurs had a remarkably bird-like brain — for example, it had reduced olfactory lobes and large, laterally displaced optic lobes. This suggests that, like modern birds, pterosaurs were usually more interested in what they could see than what they could smell. The pterosaur brain seems to have been relatively small when scaled against body mass, however, with the brains of both *Rhamphorhynchus* and *Anhangura* plotting below the limits for extant birds. Witmer *et al.* propose, convincingly, that this is primarily related to differing ancestries: birds inherited their grey matter from relatively big-brained theropod dinosaurs⁶, whereas pterosaurs inherited theirs from relatively small-brained archosaurs⁷.

The most striking results concern brain structures called floccular lobes and semi-circular canals. Floccular lobes extend outwards and backwards from the rear part of the brain and are exceptionally large in pterosaurs, while semi-circular canals encircle the floccular lobes and are involved in balance. In living vertebrates the orientation of the semi-circular canals, in particular the lateral canal, relates directly to the 'alert' position usually adopted by the head during locomotion and other activities. Exploiting this association, Witmer *et al.* show that, whereas the head posture of *Rhamphorhynchus* and probably all basal pterosaurs was normally horizontal, in *Anhangura* and most, if not all, other derived forms, the head was directed sharply downwards at about 30°.

This is an elegant piece of work. But explaining the difference

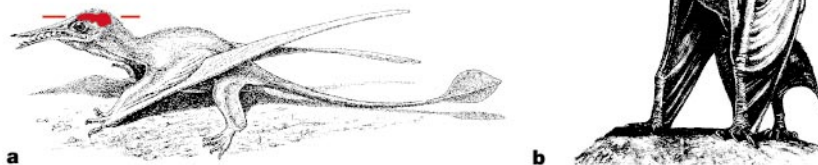


Figure 1 Ground truth? Pterosaur head orientations inferred by Witmer *et al.*², and their interpretation in terms of posture when on the ground. a, The horizontal alignment of the lateral semi-circular canal, indicated by the red line, is consistent with a crouching posture and forward-directed head in basal pterosaurs, represented by *Rhamphorhynchus*. b, In derived forms such as *Anhangura*, the reorientation of the canal can be interpreted in terms of an upright position and a downward-pointing head. (Pterosaurs redrawn from ref. 10 and not to scale.)

in head orientation is not easy. Maybe, suggest Witmer *et al.*, it relates to the large cranial crests borne by many derived pterosaurs, including *Anhanguera*, which could have affected skull aerodynamics during flight and required some repositioning of the head. But this is inconsistent with the recent discovery of large cranial crests in several basal pterosaurs^{8,9}, and their occasional absence in *Anhanguera* for instance. Alternatively, could head depression be related to feeding? *Anhanguera* and other derived pterosaurs have been interpreted as aerial fish-catchers¹⁰, a feeding style that would have benefited from a downward-directed skull — especially as it may have permitted some stereoscopy, enabling accurate judgement of distance to a moving target. Again, however, there are inconsistencies. Many derived pterosaurs, such as the flamingo-like, filter-feeding form *Pterodaustro*, were not airborne fishers. But several basal forms were, as a specimen of *Rhamphorhynchus* with a fish in its belly eloquently testifies. Yet they still fished successfully with their level heads.

A more persuasive answer to this problem lies on the ground (Fig. 1). Like their reptilian ancestors, basal pterosaurs with their relatively short arms were condemned to walk with the body and head in a near-horizontal position, aligned with the lateral semi-circular canal. By contrast, functional studies¹¹ suggest that derived forms used their relatively long arms to prop themselves upright. But because they still needed to see in front of them as they walked, this required some restructuring of the skull and its posture, one consequence of which would have been reorientation of the semi-circular canals.

Attractive as they are, these ideas do not address the extraordinarily large size of the floccular lobes in pterosaurs. Witmer *et al.* suggest that this region of the brain may have been responsible for coordination of the head, eye and neck, permitting gaze-stabilization during flight. Such an ability would have been useful for aerial hunters that relied primarily on sight. But not all pterosaurs had such a lifestyle, so this is not an entirely satisfactory explanation.

Far more convincing, in my view, is Witmer and colleagues' proposal that the floccular lobes were responsible for processing large volumes of sensory data generated by the wing membranes. This is a plausible idea, because in other vertebrates the floccular lobes receive sensory inputs from skin and muscles. New, extraordinarily well-preserved pterosaur material from Germany¹² and China¹³ shows that the wing membranes were highly complex, containing structural fibres, blood vessels and a fine network of muscles. These features would have given the wings the ability to collect and transmit sensory information about local conditions

within the membranes, enabling pterosaurs to build up a detailed map of the forces experienced by the wings from moment to moment. Processing via the floccular lobes could have allowed them to respond very rapidly, through localized contraction or relaxation of muscle fibres within the membrane and coordination with fore- and hind-limb movement. Equipped with their 'smart' wings, pterosaurs would have had excellent flight control. Despite their antiquity, they could even have outperformed modern birds and bats. ■

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Medical technology

Balancing the unbalanced

Frank Moss and John G. Milton

Elderly but healthy people are often seriously injured in falls. Exploiting the phenomenon of stochastic resonance, biological physicists have designed a shoe with a vibrating insole that helps maintain balance.

To stay upright, the human body uses exquisitely complicated and delicate feedback control, operating through the sensory and central nervous systems¹. It does so in ways similar to a performer balancing a pole on a finger², by applying minute forces and torques through muscles in the feet, ankles and knees. But these muscles must receive signals that convey information on how much force to apply through which muscles. This information is initially supplied to the central nervous system by neurons located in various joints and the soles of the feet. With ageing, the sensory thresholds of these neurons increase. The information they transmit is therefore degraded, impairing balance.

As they report in the *Lancet*³, J. J. Collins and his colleagues have successfully tested an idea about how information from the periphery of the sensory system might be enhanced. They find that the application of a random vibratory stimulation to the soles of the feet can reduce 'postural sway', and improve balance in both young and elderly human subjects. Inexpensive, easily constructed insoles, made of viscoelastic silicone gel, generated the stimuli. Three mechanical vibrators, about the size of three stacked coins, were embedded in each insole, one in the heel and two in the forefoot regions. They were driven by random voltage generators, and the resulting vibrations propagated throughout the gel.

Collins *et al.* measured postural sway by monitoring the position of reflective markers attached to each subject's shoulder using

motion-analysis cameras. They applied the vibratory stimuli at random times, unknown to the subjects, and at strengths that were slightly below each subject's threshold of perception. Analysis of tracings of the marker motion quantified the threshold at which sensory feedback mechanisms kicked in to stabilize sway, enabling the authors to statistically characterize the subjects' balance control⁴. All subjects showed clear improvement with the introduction of vibratory random fluctuations ('noise'), with the elderly group showing the largest benefit. Other studies indicate that patients whose balance is impaired by stroke or by nerve damage associated with diabetes might also benefit⁵. The insoles have not yet been tested on subjects in motion, however, nor have they actually been shown to reduce falls, for example during walking or climbing stairs.

How does the beneficial effect arise? The information content of subsensory stimuli is known to be increased by the addition of noise through stochastic resonance^{6,7}. This phenomenon is rooted in the physics of systems with thresholds, such as sensory neurons. Such systems transmit information by means of markers, or action potentials, which are generated when a threshold is crossed. Noise added to a subthreshold stimulus increases the threshold-crossing rate, thus improving the quality of the transmitted information, and the optimal noise intensity yields the maximum improvement. Much has been written on the subject (for instance, for a review focused on perception, see

ref. 8). Experimentally, the effect has been demonstrated in the tactile system^{9,10} and, as applied here, in behavioural processes in both animals¹¹ and humans¹² that depend on perceptive thresholds.

But Collins and co-workers³ did not specifically search for the optimal noise intensity — the characteristic signature of stochastic resonance. So the benefits might arise from a different cause. The upright human body behaves like an inverted pendulum that is controlled by time-delayed feedback mechanisms¹³. Thus it could be that the vibrating insoles stabilize posture by introducing random, vertical vibrations², or by making the subjects more aware of the extent of their swaying. But neither of these possibilities is likely: first, because the insoles do not induce motion of the pivot point (the ankle and foot); and second, because the stimulus is not perceptible.

Theory aside, Collins and colleagues' observations point to a potentially valuable way of tackling a serious medical problem. Falls are the most common cause of trauma, and the largest single cause of accidental death, among the elderly. Moreover, at least in the Western world, an increasing proportion of the population is entering the elderly category. Further research and development work will be needed. But if simple and inexpensive vibratory soles could reduce falls by even a small fraction, the benefits would be considerable. ■

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Neuroscience

States of mind

Dario L. Ringach

In the brains of anaesthetized animals, neurons create spontaneous patterns of activity that resemble representations of visual stimuli. This finding may change our notions about visual perception.

Every waking moment, as we experience the world through our eyes, groups of neurons in our brain fire electrical impulses to enable us to perceive our surroundings. But what happens in our brains when our eyes are closed? In the absence of visual stimulation, the region of the brain that processes this information, the visual cortex, is not 'silent' — the neurons continue to fire. Until now, it was thought that these spontaneous patterns of activity were random, but on page 954 of this issue, Kenet *et al.*¹ report that this is not so. Instead, the cortex seems to show intrinsic patterns of activity that evolve over time by switching among a specific set of states. Remarkably, these states resemble the patterns of activity that are produced in response to certain

visual stimuli. Studying how the intrinsic cortical states interact with incoming visual information might bring us closer to understanding perception.

Activity in the visual cortex depends not only on the nature of a visual stimulus, but also on the state of the cortex at the time of stimulation². This, in turn, depends on several factors, including the sequence of the preceding stimuli, the behavioural state (for example, levels of alertness or expectation) and the background pattern of brain activity that occurs in the absence of stimulation — the so-called spontaneous or ongoing activity.

Classical studies of brain function aim to measure the average behaviour of a neuron in response to a particular stimulus. In such experiments, the spontaneous activity in the

Photonics

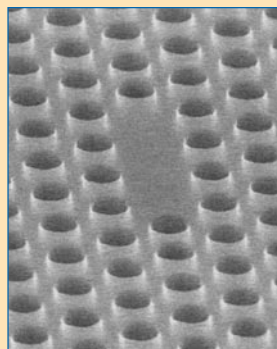
Defective quality

In most areas of technology, defects are rarely welcome. For instance, in the semiconductor material inside a computer chip, defects might trap electrons and block the flow of current. But in photonic crystals, introducing precisely engineered defects could hold the key to a range of applications, including telecommunications and quantum computing. A new principle for the design of such defects is presented in this issue, by Susumu Noda and colleagues (*Nature* **425**, 944–947; 2003).

The periodic structure of a photonic crystal forms a 'bandgap', which forbids the propagation of light within a well-defined range of wavelengths. Making defects in the structure can then capture and

confine light, creating a photonic cavity that can control light in ways that are not possible with conventional optics.

The photonic crystal investigated by Noda *et al.* consists of a thin slab of dielectric material, such as silicon, with a two-dimensional array of sub-micrometre-sized holes cut into it. There is a variety of ways to create defects in this structure, such as making one or more of the holes bigger or smaller, or removing them completely. Previous attempts at confining light in a photonic crystal have generally concentrated on the size or number of defects, or the manner in which light is coupled in and out of them. These authors, however, have investigated the



properties not of the defect holes themselves, but of those surrounding them.

First they introduced a defect in the photonic crystal with the absence of three adjacent holes (pictured). Without further

modification, this makes an abrupt interface between the defect and the surrounding array, the severity of which allows trapped light to leak out. But by shifting the position of the holes on each side of the defect, Noda *et al.* found that they could soften this interface and reduce light leakage. And by doing so by just the right amount, the confinement efficiency, or quality factor, of the defects improves by as much as 100-fold, compared with previous studies.

This approach, which the authors refer to as "confining light gently to confine it strongly", should improve the performance not only of similar two-dimensional systems but of photonic crystals in general.

Ed Gerstner

cortex at the time of stimulation is considered a nuisance — a source of noise that contributes to trial-to-trial variability^{2,3}. It is gradually becoming clear, however, that we might better understand how the cortex processes visual information if we knew what this spontaneous activity represents and how it interacts with incoming signals to generate a 'real-time' response^{2,4-6}. Kenet *et al.*¹ have taken a step in this direction by studying spontaneous activity in the visual cortex in the absence of any incoming visual stimulation.

To visualize spontaneous brain activity, the authors injected fluorescent dyes into the cortex of anaesthetized cats. The dyes are sensitive to the changes in voltage that occur when neurons are stimulated, so the intensity of the fluorescence provides a read-out of brain activity. This technique enables the average activity of thousands of neurons in a large patch of cortex (around 10 mm²) to be visualized with relatively high temporal resolution.

Kenet *et al.* find that spontaneous activity in the visual cortex is far from random. Remarkably, it fluctuates between specific states that resemble those evoked by a stimulus oriented in visual space. In other words, even without any visual input, cortical neurons fire in particular patterns, which resemble the so-called orientation maps that are produced in the cortex by looking at oriented stimuli (Fig. 1). It is known that different maps are produced by horizontal, vertical or oblique stimuli. The authors found that brain activity in the anaesthetized cats fluctuated between different orientation maps, but that the cortex spent more time 'visiting' the maps that corresponded to either horizontal or vertical orientations. This might explain why animals seem better able to recognize vertical and horizontal stimuli than oblique stimuli.

The new findings may open avenues to addressing many unsolved problems regarding cortical processing and visual perception. As the authors recognize, the next step will be to investigate whether the spontaneous cortical states have any functional significance, and what that might be. To address this issue, it will be necessary to see whether these states also occur in the brains of animals that are awake. If the findings can be replicated, we can then look at how the different spontaneous brain states interact with external stimuli to influence visual perception and performance.

One hypothesis is that the ability to recognize an oriented visual stimulus (for example, a horizontal or vertical grid) might be enhanced if the orientation of the stimulus corresponds to the spontaneous orientation map in the cortex. This could be tested by training an animal to detect different visual patterns at arbitrary orientations and measuring the cortical activity at the time the

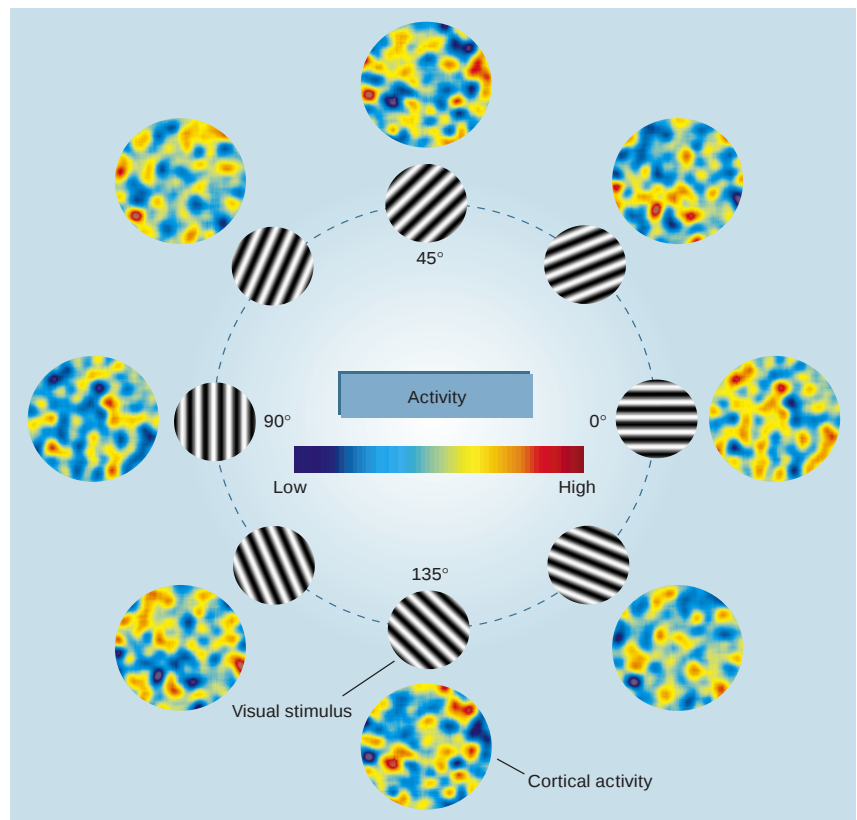


Figure 1 Mapping orientation. Each large circle shows the activity across thousands of neurons in a patch of the visual cortex of cats (the part of the brain that processes visual information) when the animals look at differently oriented stimuli. A particular 'orientation map' is produced for each different visual orientation. Kenet *et al.*¹ now suggest that these orientation maps are an intrinsic component of the brain, which are created spontaneously even when our eyes are closed.

visual stimulus is given. The animal's ability to recognize particular orientations could then be correlated with the spontaneous state of the cortex at the time the stimulus is presented.

If the ability to perceive oriented stimuli is found to depend on the cortical state^{5,6}, we can then ask a number of important questions about cortical function. Is the time the cortex spends visiting particular states a function of attention? In other words, if an animal is trained to perceive horizontal stimuli, will there be a corresponding increase in the time the cortex spends sampling the horizontal orientation map relative to the other maps? Perhaps perceptual learning has more to do with 'top-down' mechanisms (brain processes learning how to 'control' the ongoing state of the early visual cortex) rather than 'bottom-up' mechanisms (external stimuli producing dramatic and long-lasting changes in the way the visual cortex processes incoming information)⁷⁻⁹. In more general terms, one might ask if such intrinsic cortical states represent the brain's 'current hypothesis' about the state of the external world^{10,11}. In this case, the purpose of the cortical machinery would be to continuously update the current hypothesis by considering new visual information.

Perception always occurs within a context. Kenet and colleagues' findings¹ show that, to understand fully how our brains allow us to perceive the world in real time, we must investigate the context in which incoming visual signals are received and how that context interacts with the signals to produce a behavioural response. ■

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Quantum physics

Seven atoms make a superfluid

Phys. Rev. Lett. **91**, 163401 (2003)

How many atoms does it take to make a superfluid? About seven, according to Yunjie Xu and colleagues. They have watched single molecules of nitrous oxide (N_2O) spinning inside clusters of helium atoms, and find that a cluster's total inertia to rotation begins to drop once it contains more than seven helium atoms.

With fewer atoms than this, the inertia increases as more atoms are added, because they stick to the N_2O molecule and make it more ponderous. But just seven atoms are enough to produce the characteristic signature of superfluidity: a drop in viscosity owing to collective motion of the quantum particles. In the clusters, this is manifested as a decoupling of the spinning molecule from its surrounding solvent of helium atoms.

'Molecular superfluidity' of nanoscale droplets has been reported previously, not just in helium but also in hydrogen. But it was not clear just how small a superfluid cluster could be. The new results, based on spectroscopic studies of clusters with countable numbers of atoms, show that for quantum many-particle systems, seven is a crowd.

Philip Ball

Cell biology

Stretch and split

J. Cell Biol. **163**, 143–154 (2003)

In order to split in two, a cell needs extra membrane to accommodate its expansion — plus the proteins actin and myosin to squeeze the two daughter cells apart. Blake Riggs *et al.* suggest that a cellular compartment called the recycling endosome may supply both membrane and actin for the job.

The team studied cell division by looking at a comparable process that occurs in early fly embryos, where dividing nuclei are surrounded by actin- and myosin-rich membranes called furrows. The authors had previously identified a protein called Nuclear fallout (Nuf) as being required for these furrows to form. Nuf is related to the

mammalian protein Arfophilin, a key component of the recycling endosome — a fact that hinted at how Nuf might aid furrow formation.

Riggs *et al.* now find that Nuf seems to control the formation of furrows (or cell division proper) by helping the recycling endosome to deliver membrane and actin to the site where furrows form. It does this by pairing up with another essential recycling-endosome protein, Rab11 (see picture). The authors speculate that vesicles from the recycling endosome may either ferry actin to the furrow directly, or carry other proteins that organize actin once they are added to the cell membrane.

Helen Pearson

Human evolution

Robust ideas

J. Hum. Evol. **45**, 99–111 (2003)

The largest-known sample of human skeletons from the Late Pleistocene comes from a place called Kow Swamp, in what is now Victoria, Australia. The Kow Swamp people had an extraordinarily robust body form compared with that of their neighbours at Lake Mungo to the north, in New South Wales.

Researchers have long wondered whether the two populations reflected widely divergent ancestries. Tim Stone and Matthew L. Cupper suggest, however, that the robustness may have been more a response to worsening climate than a reflection of widely sundered heritage.

Recent re-dating of the Lake Mungo specimens placed them at around 40,000 years old, just before a sharp climatic trend towards aridity and glaciation in southeastern Australia. The Kow people were once thought to have lived 9,000–15,000 years ago, on the basis of radiocarbon dates. Stone and Cupper believe that those dates were due to contaminants, and, after dating sediment from the site using optically stimulated luminescence, put the age of the Kow people at around 20,000 years. This time was the coldest, driest part of the Last Glacial Maximum, and the ruggedness of the specimens' body form could have been an evolutionary response of Mungo-like people to these challenging conditions.

Henry Gee

Psychoacoustics

Hearing ghosts

Chaos **13**, 1226–1230 (2003)

When we hear two tones simultaneously, the brain often 'invents' a third one at a lower pitch. Dante R. Chialvo argues that the illusory note — which is generally the missing 'fundamental' of the two real tones, making them sound like harmonics — depends on omnipresent random noise in the signal reaching the auditory neurons.

Chialvo assumes that the missing fundamental is produced by constructive interference between the two real tones, which generates acoustic peaks at a frequency equal to the greatest common divisor of these two frequencies. But it is not quite that simple, because the amplitude of this linear combination of frequencies is too low in itself to cause auditory neurons to fire. Background noise, however, nudges the peaks above the firing threshold, in much the same way that noise can enhance detection of a signal in the phenomenon of stochastic resonance. This introduces a nonlinearity into the effect, which can account for the shift in the pitch of the missing fundamental when, for example, three equidistant stimulating tones are shifted in pitch. If the illusory frequency were set simply by the difference in frequency between these tones, such a pitch shift would have no discernible effect.

Philip Ball

Conservation

Model distributions

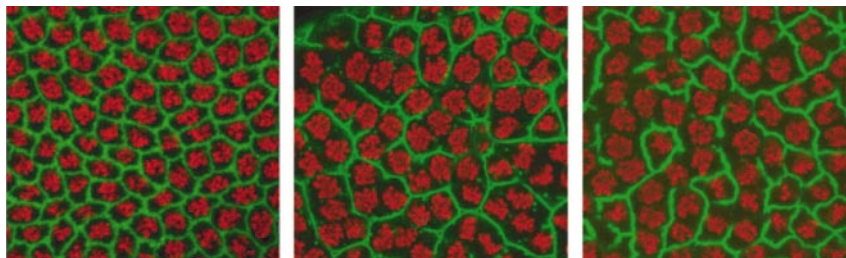
Glob. Change Biol. **9**, 1353–1362 (2003)

Understanding how climate and land-use changes will affect species distributions is a crucial task for conservation biologists. Computer models can help to predict shifts in distributions, says Wilfried Thuiller — but the opinion of human experts is still needed to validate the results.

Thuiller has developed a computational framework called BIOMOD (for biodiversity modelling) that combines four different modelling techniques. He tested it by trying to 'predict' the current distributions of 61 European tree species from climate data, and comparing the results with the observed distributions. He then projected the distributions into the future using a simulation of climate change in Europe.

The BIOMOD ensemble performed well at predicting current distributions. But taken individually, the best-performing model varied from species to species, and the differences in accuracy grew as the predictions became more ambitious. Thuiller argues that researchers could develop forecasts using the most accurate model for their preferred species, or a composite of models, backed up with expert knowledge of 'ground truth'.

John Whitfield



Plough lane: how furrows (green) should look (left), and what happens when the proteins Nuf (centre) or Rab11 (right) are mutated.

SARS virus infection of cats and ferrets

There is now a choice of animal models for testing therapies against the human virus.

The reservoir of the coronavirus isolated from patients with severe acute respiratory syndrome (SARS)^{1,2} is still unknown, but is suspected to have been a wild animal species. Here we show that ferrets (*Mustela furo*) and domestic cats (*Felis domesticus*) are susceptible to infection by SARS coronavirus (SCV) and that they can efficiently transmit the virus to previously uninfected animals that are housed with them. The observation that these two distantly related carnivores can so easily be infected with the virus indicates that the reservoir for this pathogen may involve a range of animal species.

Serological and virological studies have indicated that Chinese ferret badgers (*Melogale moschata*), masked palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*) can be infected with a virus that is very similar to SCV (ref. 3). Domestic cats living in the Amoy Gardens apartment block in Hong Kong, where more than 100 residents contracted SARS last year, were also found to be infected with SCV.

To test the susceptibility of domestic cats and ferrets to SCV infection, we inoculated them intratracheally with 10^6 median tissue-culture infectious dose units (TCID₅₀), which we obtained from patient 5688 (who died from SARS) and then passaged four times on Vero 118 cells^{4,5} *in vitro*. We then took nasal, pharyngeal and rectal swabs from the animals on different days post-infection (p.i.). Four animals from each group were killed at 4 days p.i. and were necropsied according to a standard protocol^{4,5}.

No clinical signs were seen in SCV-inoculated cats, whereas three out of six ferrets became lethargic from days 2–4 p.i. and one of these ferrets died 4 days p.i. All cats (Fig. 1a) and ferrets (Fig. 1b) shed SCV from the pharynx, starting at 2 days p.i. and continuing until days 10 and 14, respectively, as demonstrated by polymerase chain reaction with reverse transcription (RT-PCR)⁵. The virus was isolated^{4,5} from all pharyngeal swabs taken on days 2–8 p.i. and from nasal swabs taken from two cats on days 4 and 6 p.i. SCV was not detected in nasal swabs from ferrets or in rectal swabs from cats or ferrets.

Infection of the respiratory tract was evident in all animals tested: SCV was isolated from the trachea and lungs (see supplementary information). Quantification of the viral titres in lung homogenates revealed relatively low SCV titres (geometric mean $\pm$ s.d.) in the lungs of SCV-inoculated cats ($1 \times 10^3 \pm 0.51$ TCID₅₀ ml⁻¹)

compared with those in ferrets ($1 \times 10^6 \pm 0.70$ TCID₅₀ ml⁻¹). Histologically, SCV infection was associated with pulmonary lesions similar to those seen in SCV-infected macaques^{4,5}, except that they were milder, particularly in SCV-infected cats, and did not feature syncytia.

In the gastrointestinal and urinary tracts, SCV was detected by RT-PCR (see supplementary information). Follow-up of the remaining SCV-inoculated animals ($n = 2$ per group) revealed that they had all seroconverted⁵ by 28 days p.i. (neutralizing antibody titres of 40–320). Two attempts to infect suckling mice through intracerebral inoculation failed.

Non-inoculated cats (Fig. 1c; $n = 2$) and ferrets (Fig. 1d; $n = 2$) that were housed with the inoculated cats and ferrets, respectively, became infected with SCV: viral titres gradually increased from 2 days p.i. onwards, peaking at days 6–8 p.i. Neither of the cats showed clinical signs of infection, but both had seroconverted by day 28 (they had virus-neutralizing antibody titres of 40 and 160, respectively). Both ferrets were lethargic and developed conjunctivitis; they died on days 16 and 21 p.i. We established by pathological examination that the main lesions in both animals were marked hepatic lipidosis and emaciation. There was

no evidence that either of these animals died from SCV-associated pneumonia, although SCV was isolated from post-mortem lung specimens of one animal.

Our results show that ferrets and domestic cats are susceptible to experimental infection by SCV, and that the virus is efficiently transmitted to animals living with them. These species might therefore be useful as animal models to test antiviral drugs or vaccine candidates against SARS.

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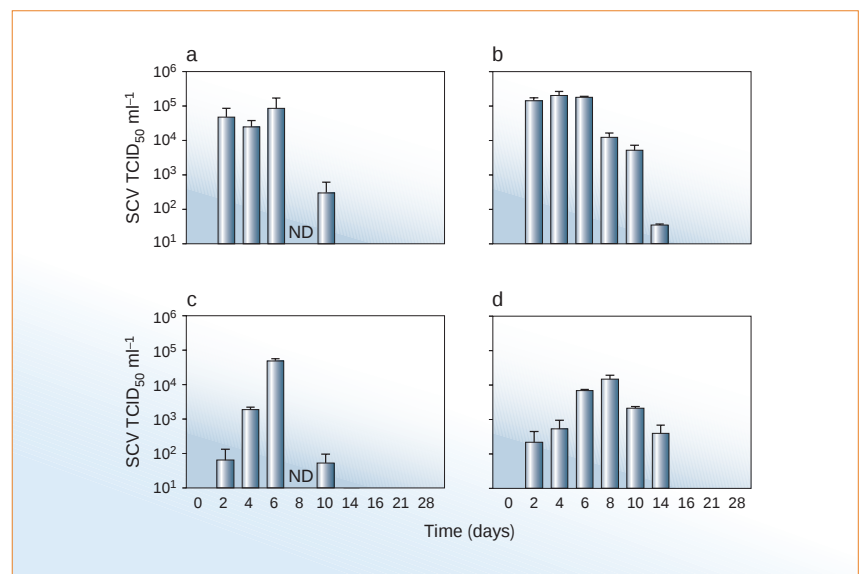


Figure 1 Daily excretion of SARS coronavirus (SCV) in ferrets and domestic cats after inoculation with the virus or exposure to infected animals. **a, b**, SCV titres per ml from cats (**a**) and ferrets (**b**) ($n = 6$ of each) that had been inoculated with SCV through the respiratory route. Four animals from each group were killed on the fourth day after infection, and two were kept until day 28. **c, d**, SCV titres from non-inoculated cats (**c**) and ferrets (**d**) ($n = 2$ of each) that had been housed with inoculated cats and ferrets, respectively. SCV excretion was quantified in pharyngeal swabs by using reverse transcription with the polymerase chain reaction, and was compared to a titrated SCV standard. ND, not determined.

Palaeontology

Preserved organs of Devonian harvestmen

Harvestmen (Arachnida: Opiliones) are a common and widespread group, the most familiar of which are recognizable by their small, rounded bodies and long, slender legs ('daddy long-legs'). Their fossil record is generally poor, but new and exceptionally well-preserved harvestmen have been found in the famous 400-million-year-old Rhynie cherts of Scotland. These remarkable and surprisingly modern-looking fossils include male and female genital structures (a penis and ovipositor) and branching tracheal tubes — providing the oldest unequivocal evidence in any arthropod for air-breathing through the tracheae.

The Early Devonian (Pragian) Rhynie cherts of Aberdeenshire are an ancient hot-springs system that preserves an early terrestrial-aquatic ecosystem¹. Both plant and arthropod cuticles have been preserved in three dimensions in extraordinary detail through floods of silica-rich water². Terrestrial arthropods from Rhynie include mites, the extinct trigonotarbid arachnids, spring-tails and myriapods³. To this list we can now add the oldest record of harvestmen. The fossils described here were discovered in thin sections of polished chert and are deposited in the palaeobotany section of the University of Münster, Germany.

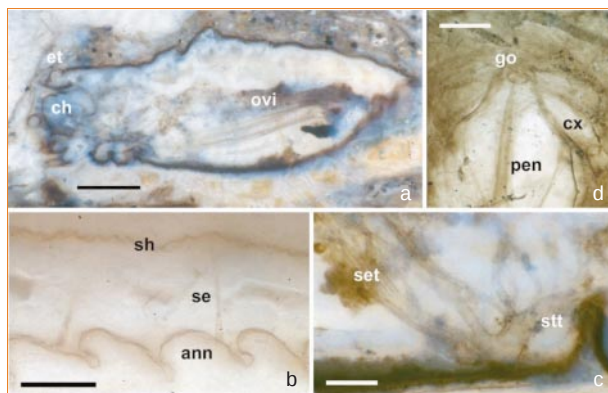
The largest harvestman (body length about 6 mm) is preserved on a slide-mounted thin section and shows a lateral section along the midline (Fig. 1a). Typically for harvestmen, the body is deep and compact in outline, with little or no tagmosis between the prosoma and opisthosoma. Many Rhynie arthropods preserve internal, cuticle-lined structures^{3,4}.

The harvestman opisthosoma contains a long, annulate feature, originating towards the back of the opisthosoma and curving gently down towards the leg bases, where it fades out of the plane of section. Comparison with living harvestmen⁵ reveals that this is an ovipositor. It is of a type that is unique to harvestmen and which, in living animals, is extended some distance beyond the body during egg-laying. The fossil ovipositor lies within a thin cuticular sheath and forms a tube of 300- μ m diameter. Surprisingly fine detail is preserved in the ovipositor itself (Fig. 1b), including its thin, deeply folded wall and several short, probably sensory, setae.

The reverse side of the same slide shows the animal at a more lateral plane of section. In the middle of the opisthosoma is a dark gut trace, and below this a series of pale, tubular structures originate from an unresolved point on the ventral surface behind the fourth leg coxa (Fig. 1c). These are clearly tracheae, because their gross morphology

Figure 1 The oldest known harvestman, from the Early Devonian Rhynie cherts of Aberdeenshire, Scotland.

a, Lateral midline section of a female specimen, revealing the outline of the body and internal ovipositor. **b**, Detail of the annulate, setose ovipositor and its membranous sheath. **c**, Reverse side of same slide, showing oldest unequivocal arthropod tracheae, with a large stem trachea and several secondary tracheae. **d**, Ventral view of male opisthosoma and posterior-most coxae, with sternites missing to reveal opisthosomal contents, including a rod-like penis. Key: ann, annulation of ovipositor; ch, chelicerae; cx, coxa; et, eye tubercle; go, genital opening; ovi, ovipositor; pen, penis; se, seta; set, secondary trachea; sh, ovipositor sheath; stt, stem trachea. Scale bars, 1 mm (a); 50 μ m (b); 200 μ m (c, d).



and position within the anterior opisthosoma are almost identical to those of living harvestmen⁶ and they show characteristic spiral thickenings near the bases of the tubes. A large stem trachea, of maximum diameter 170 μ m, curves anteriorly into the prosoma but fades rapidly out of the plane of section; four narrower, secondary tracheae run posterolaterally towards the gut trace within the opisthosoma, further dividing into about 20 individual tubes.

A second specimen is smaller (with an opisthosoma length of about 1.2 mm) and is essentially a ventral view, in which the surface of the opisthosoma has been lost during sectioning to reveal internal structures. This specimen is associated with extremely long and slender legs, more or less in the same position as in life. Other distinctive harvestman features here include the anterior displacement of the genital opening, represented by an oval ring of cuticle nestled between the coxae of the fourth pair of legs.

A smooth, slender, rod-like median structure originates towards the back of the opisthosoma and tapers towards the genital opening. By comparison with living harvestmen⁷, this is evidently a penis and the male specimen preserves the oldest fossilized example of such an intromittent organ (Fig. 1d). It is extended from the body during mating and facilitates direct sperm transfer. The shaft of the penis tapers anteriorly, where there is a disjunct transition to the tip. This is formed from an ovate, swollen glans, which merges into a slender, bluntly pointed stylus. Again, details are preserved, including short setae on the glans, an apparent intromittent canal within the stylus and possible muscle tendons attaching to the glans. An almost identical morphology occurs in closely related living forms⁷.

The most remarkable aspect of these Rhynie chert fossils is their modernity, not only in gross morphology, but in the fine spatial and structural details of their respiratory and reproductive organs. Although tracheae have been inferred from putative spiracles in a myriapodous Devonian arthropod⁸, the

harvestman structures described here provide the oldest unequivocal evidence for branching tracheal tubes in any arthropod so far. Together with the oldest book lungs, also from Rhynie⁴, these tracheae indicate that at this time that had modern-looking pulmonate and tracheate respiratory systems. The form of the genitalia imply that the modern harvestman reproductive behaviour of copulation, and subsequent oviposition in the substrate, was also established early in the Devonian.

In harvestman phylogeny⁹, four major lineages are recognized: Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores. The genitalia allow us tentatively to resolve the position of these Rhynie chert fossils. An annulate ovipositor occurs only in Cyphophthalmi and Eupnoi. The relatively large size and the presence of a median eye tubercle rules out the basal clade Cyphophthalmi; the ovipositor therefore indicates that the Rhynie harvestmen are crown-group arachnids that can be referred with some confidence to the extant suborder Eupnoi. This group includes the common and familiar 'daddy long-legs' type of harvestman.

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A gene expression atlas of the central nervous system based on bacterial artificial chromosomes

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The mammalian central nervous system (CNS) contains a remarkable array of neural cells, each with a complex pattern of connections that together generate perceptions and higher brain functions. Here we describe a large-scale screen to create an atlas of CNS gene expression at the cellular level, and to provide a library of verified bacterial artificial chromosome (BAC) vectors and transgenic mouse lines that offer experimental access to CNS regions, cell classes and pathways. We illustrate the use of this atlas to derive novel insights into gene function in neural cells, and into principal steps of CNS development. The atlas, library of BAC vectors and BAC transgenic mice generated in this screen provide a rich resource that allows a broad array of investigations not previously available to the neuroscience community.

Since the revolutionary work of Ramon y Cajal¹, the structure of the nervous system has been interpreted in the context of specific neuronal and glial cell types, and their inter-relationships. The remarkable range of cell types present in the CNS and the complexity of their interconnections complicate efforts to provide a thorough analysis of CNS gene expression, and demand that expression data be collected at cellular resolution. The development of transgenic mouse methodology for reporter gene analysis using precisely modified BACs offers a new method for correlation of gene expression with cell type, because the elaborate specializations of morphologically complex cells can be visualized^{2,3}. We describe here a large-scale project using BAC transgenic mice to provide detailed gene expression information for CNS-expressed genes, to identify a library of BAC vectors for manipulation of specific CNS cell types, and to provide a collection of mouse lines carrying enhanced green fluorescent protein (EGFP) reporter genes in a large variety of CNS cell types. We demonstrate the use of this approach to formulate detailed hypotheses concerning individual gene functions, to analyse cell migration, to discover novel topographic features of CNS projections, and to define BAC vectors that provide experimental access to cells within the circuitry of neural systems.

The GENSAT BAC Transgenic Project

The ability to use reporter gene strategies in transgenic mice to map gene expression at cellular resolution was first demonstrated using modified BACs carrying ~150 kilobases (kb) of genomic DNA surrounding the mouse *Zipr1* gene³. Subsequent studies have established the general utility of BAC transgenic mice for analysis of mammalian gene expression and function, for the isolation of specific cell populations, and for the identification of effective vectors that can allow reproducible experimental access to specific cell types². Visualization of EGFP expression in specific neuronal or glial cell populations has also provided compelling evidence that this strategy can result in the identification of expressing cell types, as the detailed morphology of EGFP-expressing cells is

apparent. The Gene Expression Nervous System Atlas (GENSAT) BAC Transgenic Project is designed to take advantage of these features of EGFP reporter genes to provide detailed expression maps for thousands of genes. The basic strategy for the project is to: (1) select genes to be analysed; (2) prepare modified BACs in which an EGFP reporter gene is substituted for the gene of interest; (3) prepare transgenic lines carrying each reporter construct; (4) analyse systematically reporter gene expression in these lines during development of the CNS; and (5) annotate the expression data and report to a public database.

Methodological considerations

This project has required critical decisions regarding the overall strategy of the project, refinements of existing methodologies, and development of new resources for data management and presentation. In particular, new methodology was developed and tested for the production of BAC reporter gene constructs, for efficient production of BAC transgenic mice, and for presentation of the anatomical data using a web-administered database (<http://www.gensat.org/>). These decisions and methodological improvements are described in the Supplementary Information.

The GENSAT BAC Transgenic Project is the first large-scale effort to use both the mammalian genome sequence and the BAC clones that provide the basis of the genome physical map to generate anatomical data and experimental resources for use by the neuroscience community. The usefulness of the GENSAT data, vectors and animals to the neuroscience community depends in part on the accuracy of the expression data obtained and the ability to target gene expression in a reproducible manner using the BAC vectors described in the GENSAT project. Comparative analysis of data collected for known genes targeted using BACs selected from the mouse genome databases versus expression data available in the literature or in our laboratories has highlighted several important points regarding the BAC transgenic approach to gene expression mapping.

First, in each of the BAC transgenic vectors, the endogenous messenger RNA and protein coding sequences have been replaced by sequences encoding the EGFP reporter gene. As in any gene-replacement experiment, the stabilities of the reporter gene mRNA and protein can be different from those of the endogenous gene products. Thus, our results identify the relative rates of transcription for each gene in cells of the nervous system; they are not a direct measure of mRNA accumulation or protein abundance for the endogenous gene products. Furthermore, the increased sensitivity of the reporter gene assays, particularly in BAC transgenic lines carrying multiple copies of the BAC transgene, may allow detection of sites of expression that are not evident from *in situ* hybridization experiments. For these and other reasons, one can expect some differences between the GENSAT expression data sets and *in situ* hybridization or immunohistochemical data, particularly for dynamically regulated genes.

Second, to achieve a high rate of success in reproducing the expression pattern of a gene of interest, it is necessary to include the entire transcription unit and all of its associated regulatory information. There is at present no way of predicting the locations of these regulatory elements. However, as the average transcription unit for mammalian genes is less than 100 kb, and the carrying capacity of BACs is several hundred kilobases, it is possible in most cases to identify a BAC covering the transcribed unit and including 50–100 kb of 5′- and 3′-flanking intergenic DNA. In our experience so far, ~85% of BACs chosen using these general parameters express reproducibly in multiple transgenic lines. Those that do not express reproducibly owing to position effects on integration into the genome are easily identified, because the sites of expression vary significantly between lines.

Third, the release of the mouse genome databases^{4,5} has markedly

improved the ability to identify and target an appropriate BAC for this screen. For example, our original BAC transgenic lines for choline acetyltransferase (*Chat*) were prepared before release of the genomic databases using a BAC (RP23-51F19) that we subsequently learned contained ~85 kb of 5′-flanking DNA, but truncated the gene ~25 kb before the polyA addition site. As shown in Fig. 1a, this BAC expressed well in transgenic mice, correctly revealing the main cholinergic brainstem nuclei (Fig. 1a, panels 4 and 5) and spinal cord motor neurons (Fig. 1a, panel 6). However, no expression was detected in the basal forebrain (Fig. 1a, panels 2 and 3), and moderate ectopic expression of the BAC transgene was detected in layer 2–3 of the cerebral cortex (Fig. 1a, panel 1). On release of the genomic databases, BAC transgenic reporter lines were redone using BAC (RP23-431D9) covering the entire *Chat* locus, including ~85 kb of 5′-flanking DNA and ~60 kb of 3′-flanking DNA. As illustrated in Fig. 1b, expression of the reporter gene in these mice faithfully reproduced that of the endogenous gene, revealing the appropriate brainstem cholinergic nuclei (Fig. 1b, panels 4 and 5), spinal cord motor neurons (Fig. 1b, panel 6), and basal forebrain (Fig. 1b, panels 2 and 3) and cortical interneuron populations (Fig. 1b, panel 1). Critical regulatory elements must reside within the 3′-flanking DNA of the mouse *Chat* locus.

Fourth, for very large genes (>200 kb), the BAC approach to gene expression mapping has a low probability of success. Thus, about 10% of the lines we have analysed so far were for large genes in which the reporter gene construct contained ~100 kb of 5′-flanking DNA and ~100 kb of the transcription unit. In only one case has this approach been successful.

Finally, for most genes, multiple copies of the EGFP reporter gene are required for a sufficient signal-to-noise fluorescent ratio in tissue slices.

Thus, multiple lines carrying the BAC transgene were routinely screened to choose an optimal line for in-depth expression analysis.

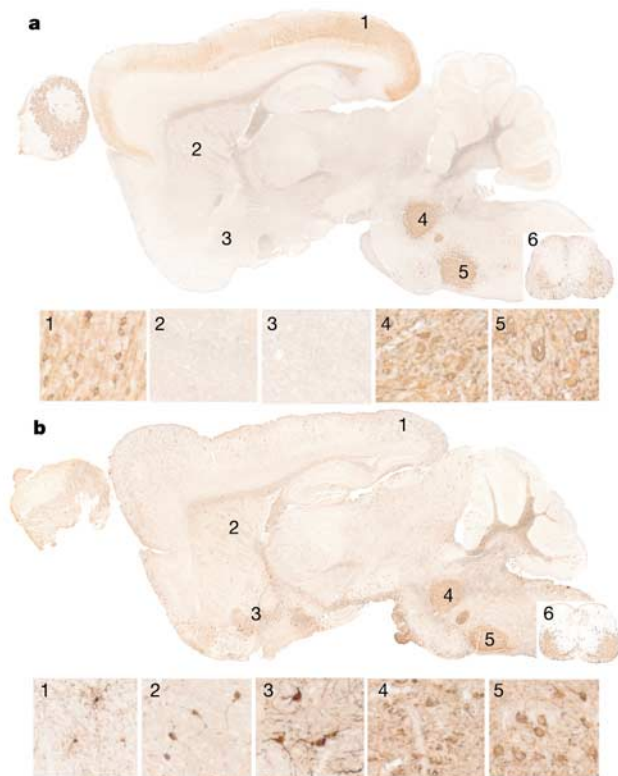


Figure 1 Expression of *Chat* from different BAC constructs. Expression in adult sagittal sections from a BAC transgenic line prepared using BAC RP23-51F19 (**a**) or BAC RP23-431D19 (**b**). Panels 1–6 show higher-magnification images of the cerebral cortex (panel 1), basal forebrain (panels 2 and 3), brainstem (panels 4 and 5) and spinal cord (panel 6).

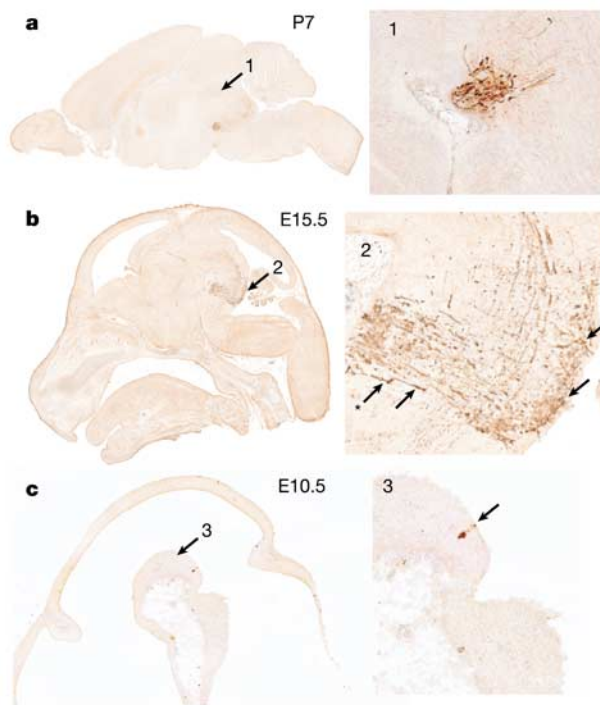


Figure 2 Cell lineage revealed by expression data from reporter gene analysis in BAC transgenic mice. **a**, Labelling of P7 *Gsc*/BAC transgenic brain; the only structure labelled is the IPN of the brainstem (panel 1). **b**, *Gsc*/BAC expression at E15.5. Higher magnification reveals individual migrating neurons (panel 2). **c**, Labelling of E10.5 reveals two labelled cells (panel 3). Higher magnification shows the two committed progenitor cells of the IPN.

Data from the chosen line are included in the database using the following descriptors: 'confirmed' refers to genes in which multiple lines yield matching data sets that agree with the available literature, or in which a single line has been produced that matches the available literature. In those cases where there is a discrepancy between the literature and the reporter gene data (missing or additional sites of expression), this is noted in the database; 'to be confirmed' refers to genes in which multiple lines produce matching data sets, but for which no independent expression data are available. 'Anomalous' refers to data collected from BAC reporter constructs producing different expression patterns in different lines (that is, position effects).

Biological insights from GENSAT expression data

To illustrate the advantages of systematic analysis of gene expression using BAC transgenic reporter analysis to formulate precise hypotheses concerning gene function in development, we have chosen specific examples relevant for cell specification, axon–target interactions, cell migrations and studies on neural systems.

The *Gsc1* gene is involved in neural specification

One of the most striking advantages of the BAC constructs is the ability to visualize small cohorts of cells that express a particular gene and to follow their development over time. *Gsc1* (goosecoid-like) was discovered during genomic analysis of the DiGeorge syndrome/velocardiofacial syndrome (DGS/VCFS) critical region in the human genome⁶. It is a member of a small family of homeodomain genes that includes goosecoid and *GSX*, and binds a conserved DNA sequence consistent with its proposed function as a transcription factor⁷. Inspection of BAC transgenic reporter lines

for *Gsc1* at postnatal day 7 (P7) reveals robust expression in a subset of neuronal cell bodies within the interpeduncular nucleus (IPN) (Fig. 2a, panel 1). Lower levels of axonal expression (and an occasional cell soma) are evident in the tegmental nucleus, which receives afferent input from the IPN (data not shown). At embryonic day 15.5 (E15.5), *Gsc1* expression in the developing IPN and in the ventricular zone adjacent to the developing IPN is evident, as are profiles of migrating neurons transiting from the ventricular zone to the area of the presumptive IPN (Fig. 2b, panel 2). The exquisite specificity of *Gsc1* expression in the developing CNS, and the ability of BAC transgenic reporter gene studies to reveal this specificity, are most dramatically illustrated by analysis of these mice at E10.5 (Fig. 2c, panel 3). Only two cells expressing EGFP were detected in serial sections of a whole *Gsc1* BAC transgenic embryo. These cells are present in the ventricular zone at the border of the developing mesencephalon and metencephalon, as expected from visualization of the expanding IPN progenitor pool in E15.5 embryos. These results suggest that *Gsc1* is a lineage marker for a specific class of neurons that arise in the ventricular zone at E10.5, establish a progenitor pool that is still differentiating and migrating into the presumptive IPN at E15.5, and eventually settle into the IPN during the first postnatal week. Although previous *in situ* hybridization data are consistent with this proposal, the ability to visualize *Gsc1*-expressing cells and their projections with such precision has allowed us to suggest a hypothesis that could not be formulated based on the *in situ* hybridization data. Furthermore, the identification of *Gsc1* expression in a small population of neurons in the IPN suggests a functional role for *Gsc1* that has not been tested in any of the studies done to assess the consequences of loss of *Gsc1* function *in vivo*. For example, loss of this small cell population as a

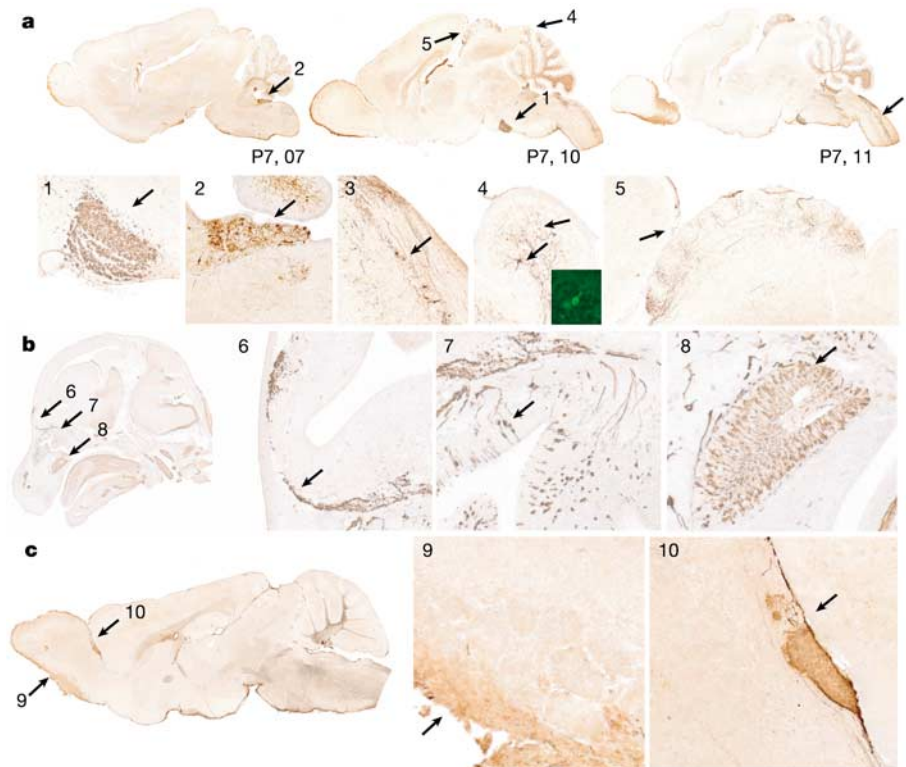


Figure 3 Predicting developmental functions for specific genes on the basis of expression data in BAC transgenic mice. **a**, Serial sagittal sections of P7 *Sema3b* BAC transgenic mice. Numbered arrows indicate areas shown in the corresponding panels. Neurons that project mossy fibre afferents to the cerebellar cortex and cerebellar Golgi neurons are labelled: pontine nucleus (panel 1), vestibular nucleus (panel 2), the spinocerebellar tract (panel 3) and the cerebellar cortex (panel 4). Confocal microscopy of Golgi neurons

(panel 4, fluorescent insert). A novel topographic map of retinal ganglion axons is seen in the superior colliculus (panel 5). Confocal microscopy reveals labelling of cells in the cerebellar cortex (panel 4, fluorescent insert). **b**, *Sema3b* expression in the developing olfactory system (E15.5): MOB (panel 6), olfactory epithelium (panel 7), vomeronasal organ (panel 8). **c**, *Sema3b* expression in the adult: external plexiform layer (panel 9); AOB (panel 10).

consequence of *Gscl* deletion would be missed unless specific markers that identify this cell type were examined in the knockout animals. Given the important functions of the habenular/interpeduncular system in the generation of hippocampal theta rhythms and the control of rapid-eye-movement sleep⁸, one might expect electroencephalographic recordings in freely behaving, *Gscl* null mutant mice to reveal important, yet subtle, behavioural phenotypes. These studies would now seem warranted, particularly given the presence of this gene in the minimal DGS/VCFS critical region⁶ and the clinical complexity of this syndrome⁹. Further investigation of these issues will be aided by the ability to visualize the EGFP-expressing neurons of the IPN using the BAC transgenic line presented here.

The *Sema3b* gene is involved in axon–target-interactions

Another key step in the development of the CNS is the outgrowth of specific sets of axons and their interaction with particular targets. In our screen, the semaphorin 3B (*Sema3b*) BAC transgenic mice provide an example of the ability of BAC constructs to reveal the morphology and axon–target projection patterns of neurons that express a particular guidance molecule. *Sema3b* is a member of a small family of secreted semaphorins, first characterized as repulsive axon guidance signals^{10–12}. Although the specific roles of *Sema3b* in vertebrates are not yet clear, *Sema3b* can act to repulse axons expressing neuropilin 2 (*Npn2*), and can antagonize the repulsive action of *Sema3a* on axonal growth cones expressing neuropilin 1 (*Npn1*)¹³.

In the developing cerebellar system *Sema3b* marked the projection neurons from the pontine nucleus, the vestibular nucleus and the spinocerebellar tract (Fig. 3a). Mossy fibres emanating from the pons (Fig. 3a, panel 1), the vestibular nuclei (Fig. 3a, panel 2) and the spinocerebellar tract (Fig. 3a, panel 3) were labelled clearly (panel 4). In addition, the large Golgi interneurons (Fig. 3a, panel 4, lower arrow and fluorescent inset) present in the granule cell layer also expressed the EGFP reporter. This distribution is particularly interesting, as each of the *Sema3b*-expressing cell types in the brainstem, spinal cord and cerebellum provide presynaptic contacts

to cerebellar granule cells. Moreover, it is noteworthy that *Sema3a* is expressed in cerebellar Purkinje cells and can collapse growth cones of basilar pontine axons *in vitro*¹⁴. As mossy fibres grow through a field of immature granule cells and Purkinje cells before establishing synapses with granule neurons, these data suggest that *Sema3b* might transiently antagonize the repulsive action of *Sema3a* during targeting of *Sema3b*-expressing mossy fibre axons to, and ramification of Golgi cell axons within, the developing cerebellar internal granular layer.

Visualization of the expression of the EGFP reporter in *Sema3b* BAC transgenic mice also predicts an important role for *Sema3b* in the formation of the circuitry of the olfactory system. In E15.5 embryos, *Sema3b* expression in the developing olfactory system is widespread (Fig. 3b). Individual cells expressing the reporter are evident throughout the olfactory epithelium (Fig. 3b, panel 7), and labelled axon fascicles extend from these cells to the surface of the developing main olfactory bulb (MOB; Fig. 3b, panel 6). Intense expression is also present in the developing vomeronasal organ (VNO; Fig. 3b, panel 8). Two features of the staining patterns suggest specific roles for *Sema3b* in the formation of axonal projections to the MOB and the accessory olfactory bulb (AOB) from the sensory epithelia. First, we do not observe *Sema3b*-expressing axons exiting from the external plexiform layer into the glomerular layer of the MOB at any stage of development (Fig. 3b, panel 6; Fig. 3c, panel 9). Given the strong expression of *Sema3a* in the developing olfactory epithelium (Fig. 3b, panel 7), it is possible that transient *Sema3b* expression is required to antagonize the repulsive effects of *Sema3a* on olfactory sensory neuron growth cones as they exit from the nasal epithelium and project to the MOB. In both the MOB and the cerebellar cortex, we suggest that *Sema3b* must be turned off as axons reach their targets so that *Sema3a* expression in mitral cells and Purkinje cells, respectively, can prevent axons from overshooting their normal target fields. Second, *Sema3b*-expressing neurons in the VNO (Fig. 3b, panel 8) project specifically to the posterior AOB (Fig. 3c, panel 10). In *Npn2* knockout mice, axons from apical VNO neurons overshoot their normal terminal field in the anterior AOB, and invade the posterior

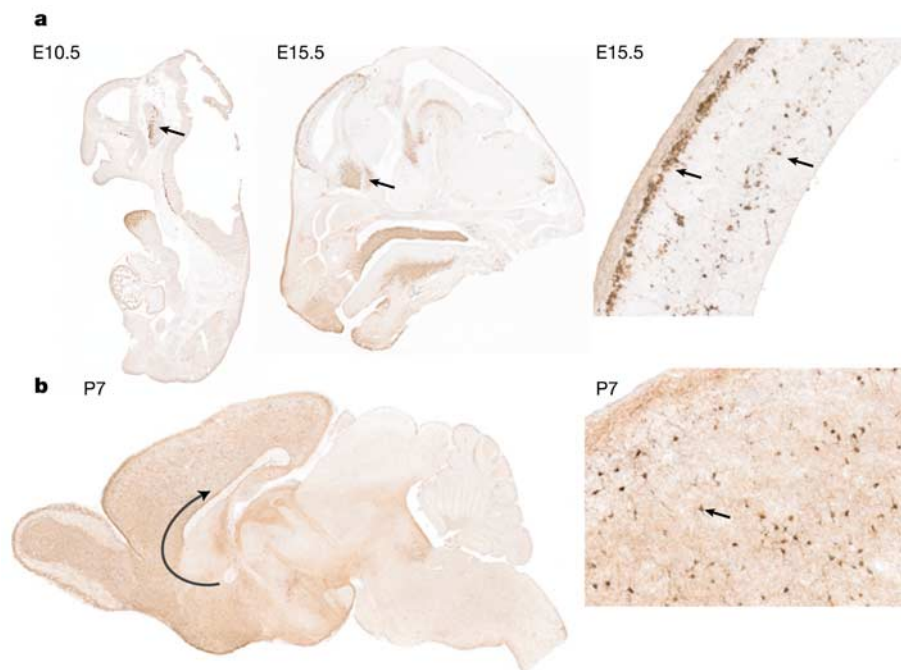


Figure 4 Genesis and migration of cortical interneurons is revealed with *Lhx6* BAC transgenic mice. **a**, Interneurons are born in the ventral forebrain (E10.5, left panel, arrow), which gives rise to the MGE (E15.5, middle panel, arrow). By E15.5, interneurons

arrive in the cerebral cortex (right panel, arrows). **b**, At P7, interneurons have spread through the cortex (arrows); higher magnification is shown in the right panel.

AOB¹⁵. As *Sema3b* can bind to *Npn2*-containing receptors with high affinity, these results suggest that the release of *Sema3b* from axon terminals in the posterior AOB may have a direct role in repelling *Npn2*-expressing axon terminals from the posterior AOB.

Finally, the *Sema3b* BAC transgenic mice revealed a novel pattern of axon–target interactions in the superior colliculus (Fig. 3a, panel 5). Axons from the retina arrive at the superior colliculus embryonically, although their invasion of the superficial grey layer is delayed until shortly after birth^{16,17}. During the first few postnatal weeks, significant remodelling of retinocollicular projections occurs to establish the adult retinotopic map^{18,19}. *Sema3a* (collapsin 1) has been shown to bind to retinotectal fibre tracts in chick embryos, and to induce retinal ganglion cell (RGC) growth cone turning *in vitro*¹⁵, suggesting a role for semaphorins in establishment of the retinotectal (retinocollicular) projection. The pattern of *Sema3b*-expressing RGC axons in the superior colliculus at P7 is provocative (Fig. 3a, panel 5). In medial sagittal sections from *Sema3b* BAC transgenic mice, an interesting striped pattern of RGC projections to the superior colliculus is evident (Fig. 3a, panel 5). These results predict a role for *Sema3b* in the formation or refinement of the retinocollicular map, and reveal a novel topographic feature of the map that is not explained by the reported actions of ephrins^{20–22} and Eph receptors in establishing the anterior–posterior map of RGC terminal fields on the superior colliculus. Taken together, the *Sema3b* expression data illustrate the advantages of reporter gene assays to reveal novel topographic features of the CNS that cannot be visualized by conventional methodology. Moreover, the *Sema3b* BAC transgenic animals offer the means to examine the behaviour of growing axons in real time as well as to carry out further genetic studies on the role of *Sema3b* in axon guidance.

Lhx6 and *Pde1c* genes reveal tangential migratory patterns

The migration of immature neurons from germinal zones to positions where they form synaptic connections is one of the hallmarks of cortical development. The BAC transgenic mice provide a means to visualize these migrations in real time and to follow the same cells over the developmental epoch of neural layer

formation. Genetic analysis of mice lacking the transcription factors *Dlx1*, *Dlx2* and *Nkx2.1* has identified an important pathway of cortical interneuron migration that runs from the basal forebrain into the cortex^{23,24}. An early population of cells migrates from the medial ganglionic eminence (MGE) up into the cortex, giving rise to GABA (γ -aminobutyric acid)-containing interneurons. Later, cells from the lateral ganglionic eminence (LGE) migrate dorsally into the cortex, also generating GABAergic interneurons. As some 20% of the neurons in the cortex are these GABAergic interneurons, these ventrodorsal cell migrations provide a large proportion of cortical neurons²⁵. Recent studies indicate that antibodies against *Lhx6* stain migrating MGE cells. In the current screen, *Lhx6* provided a marker for these cells²⁶. Although antibody labelling studies showed expression of *Lhx6* in this migratory stream, they did not provide direct access to the development of cells expressing *Lhx6*. As shown in Fig. 4, *Lhx6*-positive cells were visible in the ventral forebrain as early as E10.5, with a large population of cells evident in the MGE by E15.5. At E15.5, cells can be seen migrating from the MGE up into the cortex. Higher-magnification views at E15.5 reveal small, labelled cells in the cortex in the marginal zone and scattered throughout the cortical plate. This pattern is in good agreement with studies of the migration pattern of DiI-labelled cells. By P7, the migration is nearly complete and labelled cells are absent from the marginal zone. Dense bands of EGFP–*Lhx6* cells are seen across the cortex; on the basis of their morphology, these are apparently interneurons. Thus, the *Lhx6*BAC transgenic mouse provides an ideal system for dynamic studies on the genesis and migration of cortical interneurons.

In addition to generating lines of mice that confirm existing models of cell migrations in the cortex, lines were identified that revealed new pathways of migration. One of these was the phosphodiesterase 1C (*Pde1c*) gene, a cyclic nucleotide-hydrolysing phosphodiesterase that is important in regulating some cyclic AMP responses of postsynaptic receptors²⁷. Although *Pde1c* has not been studied extensively in the CNS, experiments in smooth muscle cells support a role in cell proliferation and differentiation²⁸. In the E10.5 embryo, staining was seen along the dorsal ridge of the



Figure 5 Expression of *Pde1c* reveals a novel and distinct migratory pathway in developing brain. **a**, At E10.5, EGFP-positive cells are visible along the dorsal aspect of the neural tube. At E15.5 (middle panel), labelled cells are present in the anterior portion and superficial aspect of the cortex. At higher magnification (right panel), cells above the cortical plate are labelled. **b**, At P7, cells in layer 1 of the cortex are labelled (arrow). The

right-hand panel shows higher magnification with confocal microscopy (inset); layer 1 Cajal Retzius cells express the reporter gene. Cells that undergo a similar pattern of migration, the cerebellar granule cells, are also labelled, as are cells in the olfactory bulb and pontine nucleus.

spinal cord, within the rhombic lip of the midbrain–hindbrain territory and in the anterior aspect of the telencephalic vesicle (Fig. 5). By E15.5, *Pde1c* marked a major population of dorsally migrating neurons, the external germinal layer of the fourth ventricle, and another population of dorsally migrating cells in the cerebral cortex. Along the fourth ventricle, the labelled cells were the precursors of the cerebellar granule neurons, which spread across the surface of the cerebellar anlagen at this developmental stage as a population of mitotic, migratory cells. In the cortex, *Pde1c* marked a population of cells in the retrobulbar region of the cortex. By E15.5, labelled cells had spread across the cortical surface in a tangential migratory pattern that matched the pattern proposed for neurons in the subpial granular layer (SGL). Although earlier evidence held that these cells were present only in human cortex, recent studies²⁹ have proposed that this cell population migrates across the surface of the cortex, intermingling with ‘pioneer’ neurons that arise from the cortical ventricular zone and migrate radially to form the marginal layer³⁰. Previous studies have not clearly distinguished these two populations as they both express the reelin gene³¹ and only occasionally calretinin. By P7, *Pde1c*-expressing cells persisted in layer 1 of the cortex, suggesting they are the SGL cell population because these cells persist longer than

pioneer neurons, which are thought to disappear by birth. Thus, *Pde1c* marks this previously unstudied cell population and provides a means to study the dynamics of the proliferation and migration of these cells.

Access to cells within specific synaptic circuits

In addition to revealing developmental processes, many of the genes studied in the BAC transgenic screen marked specific cell classes within particular systems of the CNS. This is one of the most important results of the screen, as EGFP-labelled cells can be used directly for anatomical, physiological and genetic analysis of the particular cell population. For example, the EGFP-labelled BAC transgenic mice provide reproducible access to specific cell populations for electrophysiological studies of CNS cells and circuits, for vital imaging, or for a variety of cell isolation and tissue culture studies. The cerebral cortex is an important example, where the ability to impale and/or image live cells can allow more detailed studies on both the nature and plasticity of cortical circuits, and the characterization of specific and functionally distinct populations of pyramidal cells and interneurons. Although the cellular composition of the striatum is significantly less complex than that of the cerebral cortex, experimental access to each of the striatal cell types can significantly accelerate studies of striatal physiology, and the pathophysiological events reproduced in mouse models for Huntington’s and Parkinson’s diseases. We have chosen the cerebral cortex and striatum as examples of the ability of BAC transgenics to provide access to specific neurons within CNS circuits.

The cerebral cortex consists of six cell layers containing pyramidal cell neurons (the primary circuit neurons, layers 2–6) and interneurons (the local circuit neurons), as well as several glial cell types. Analysis of the first 100 genes screened in the GENSAT project revealed both layer- and cell-type-specific expression in the developing and adult cerebral cortex. For example, specific subsets of layer 2–3 pyramidal neurons are labelled in semaphorin 6D (*Sema6d*) BAC transgenic mice (Fig. 6a). *Sema6d* is a recently identified member of the semaphorin gene family whose expression and functions in the mouse CNS are not known. At P7, labelled cells in layer 2–3 have completed their radial migrations from the collapsing ventricular zone of the telencephalon, and are in the process of forming their connections with other cortical neurons. A novel gene, *P271*, is expressed predominantly in layer 2 and 5 pyramidal cells (Fig. 6b). This is a novel protein with a single proline-rich domain that is closely related to the human *KIAA1399* gene. The morphology of expressing cells is clearly evident, including the ramifications of basal dendrites within layer 5 and the extension of the apical dendrite and its elaboration within layer 1 of the developing and adult cortex. Inspection of the *P271* data sets also reveals staining of axons as they pass through the striatum *en route* to subcortical targets. Dopamine receptor D4 (*Drd4*) BAC transgenic lines express at high levels in the prefrontal cortex (Fig. 6c). At high magnification, these cells are identified as layer 5 pyramidal cells. This is consistent with previous data showing enrichment of this receptor in the prefrontal cortex, a principal target for antipsychotic drugs³². In contrast to the examples presented above, *Otx1* is expressed in most layer 5 and 6 pyramidal cells throughout the developing and adult brain, as previously reported^{33,34}. In this case, the very high levels of *Otx1* expression in the developing and adult cerebral cortex, and the presence of EGFP in the dendritic processes of layer 5 and 6 pyramidal cells extending throughout the cerebral cortex, prevents effective visualization of cell somata by diaminobenzidine immunohistochemistry. To present convincing evidence of the restricted expression of *Otx1* in the correct cortical layers, direct confocal imaging is used to reveal the morphology and distribution of expressing cells in the *Otx1* BAC transgenic line (Fig. 6d, right panel). The presentation of data from both immunohistochemical and direct confocal imaging of the EGFP reporter gene in these lines ensures that one can

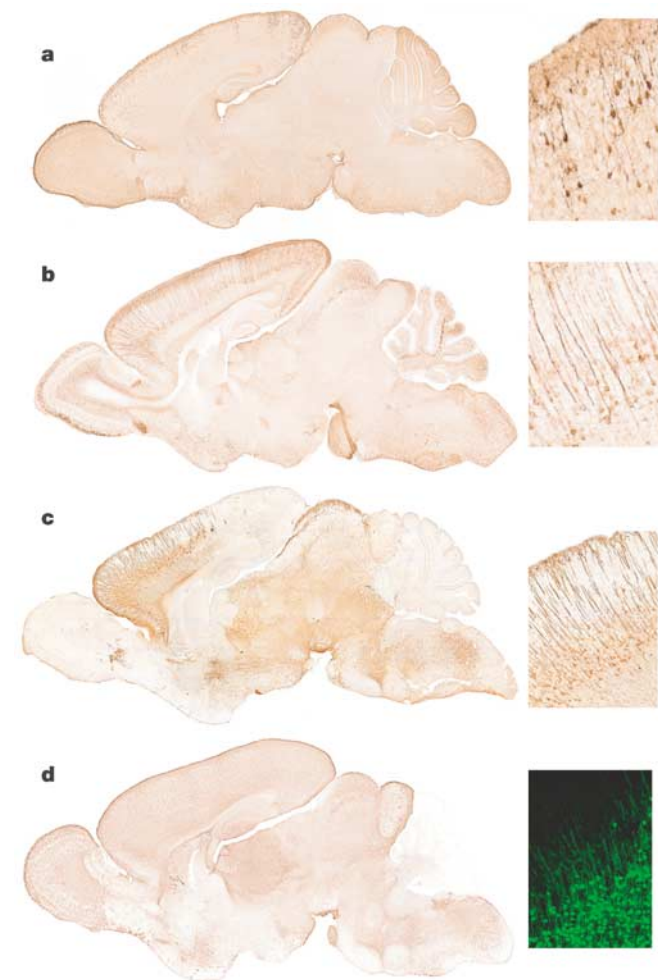


Figure 6 Layer-specific gene expression in the developing cerebral cortex. **a–d**, P7 BAC transgenic mice expressing *Sema6d* (**a**), *P271* (**b**), *Drd4* (**c**) and *Otx1* (**d**). The right-hand panels show higher magnification of cells in the same sections. *Sema6d* is expressed in cells of layer 2–3. *P271* is expressed in pyramidal neurons of layers 2 and 5. *Drd4* is expressed in layer 5 pyramidal cells in the frontal cortex, and *Otx1* is expressed in pyramidal cells of layer 5–6. For *Otx1*, pyramidal cells are evident in the confocal image of unstained sections (**d**, right-hand panel).

properly assess the specificity of expression for all BAC transgenic lines. These data identify BAC vectors and transgenic animals that provide unprecedented tools for further analysis of the development, function and degeneration of the mouse cerebral cortex.

The striatum, a non-layered structure, is the largest component of the basal ganglia. Striatal projection neurons, approximately 90% of the neuronal population, provide the output from the striatum and receive nearly all of the input from extrinsic afferents and from striatal interneurons. The small population of striatal interneurons is thought to regulate afferent activity within the striatum³⁵. The development of this system is complex, with cells deriving principally from the LGE and MGE. In the adult, all of the psychomotor behaviours ascribed to the striatum are linked to the dopaminergic innervation from the substantia nigra and the ventral tegmental area. Similarities in the morphology and neurochemistry of the cells in the striatum have confounded studies on the development of the neurons and their target interactions. In the BAC transgenic screen,

several genes revealed the cell classes and projection patterns of the striatum. As illustrated in Fig. 7a, medium spiny neurons projecting to the globus pallidus are labelled in the dopamine receptor D2 (*Drd2*) BAC transgenic mice^{36,37}. In dopamine receptor D1a (*Drd1a*) BAC mice (Fig. 7b), medium spiny neurons projecting to the substantia nigra are labelled. Anatomical data suggest that the *Drd2* EGFP cells express enkephalin, whereas the *Drd1a*-positive cells express substance P^{36,38}. Functional studies on acutely isolated EGFP-labelled neurons can now clarify whether there is co-localization of the two dopamine receptors in a subset of medium spiny neurons. A subpopulation of medium spiny neurons projecting to the substantia nigra, presumably also expressing *Drd1a*, expresses the M4 muscarinic cholinergic receptor (*Chrm4*) (Fig. 7c). These data are in agreement with electron microscopic studies in rats showing *Chrm4* localization to the somata and dendrites of medium spiny neurons³⁹. The distribution of the *Chrm4*-expressing subpopulation across the striatum, and their specific localization to the striatal matrix, is of particular interest. Large, cholinergic aspiny neurons of the striatum are specifically labelled in the *Chat* BAC transgenic mice (Fig. 7d). Finally, clusters of medium spiny neurons that project to the substantia nigra are revealed in prodynorphin (*Pdyn*) BAC transgenic mice (Fig. 7e). These cells correspond to the striatal 'patches' that have been documented in a number of anatomical studies of the developing and adult striatum⁴⁰. Taken together, this set of BAC transgenic mice reveals the principal cell types and projection pathways of the striatal system. These mice offer new tools for the analysis of the development of the striatum, and for a clearer understanding of how the dopamine and cholinergic environments influence synaptic function of specific subclasses of striatal neurons. Thus, they should facilitate novel neurophysiological and cell degeneration studies critical for our understanding of Parkinson's disease as well as neuropsychiatric disorders including Tourette's syndrome, schizophrenia and drug addiction.

Implications for biological research

We report here selected findings from an ongoing large-scale screen of CNS gene expression in BAC transgenic mice. The screen uses novel vectors for the preparation of BAC reporter gene constructs, as well as methods for high-throughput transgenesis, histology and microscopy. Moreover, a MySQL database of the full-scale TIFF images, which can be viewed in browser format online (<http://www.gensat.org/>), has been generated to allow public access to the GENSAT data and for the design of further experiments. The information and materials generated so far provide immediate opportunities for further investigation. However, the full impact of this project will not be realized until many more reporter gene mice are analysed, and until the reagents and information from this screen are combined with further methods for genetic manipulation of the mammalian CNS².

The use of BAC transgenic reporter genes to study gene expression and provide experimental access to specific cell types can stimulate many fields of biological research. Thus, the capabilities for cell-specific genetic and physiological experimentation in the CNS that issue from these studies can be readily extended for the analysis of other cells, organs or tissues expressing the genes chosen for analysis by GENSAT, or in the context of similar future efforts. Given the general utility of this approach, there are several issues that warrant further discussion.

First, the use of a reporter gene that can reveal the morphology of complex cells and the systematic analysis of gene expression is necessary to obtain detailed insights and formulate precise hypotheses about gene function or development. The ability to present these large data sets from a systematic analysis for the benefit of other scientists is critically dependent on the use of a web-based browser that can present the captured data at full resolution. Although generation of large numbers of transgenic or gene-

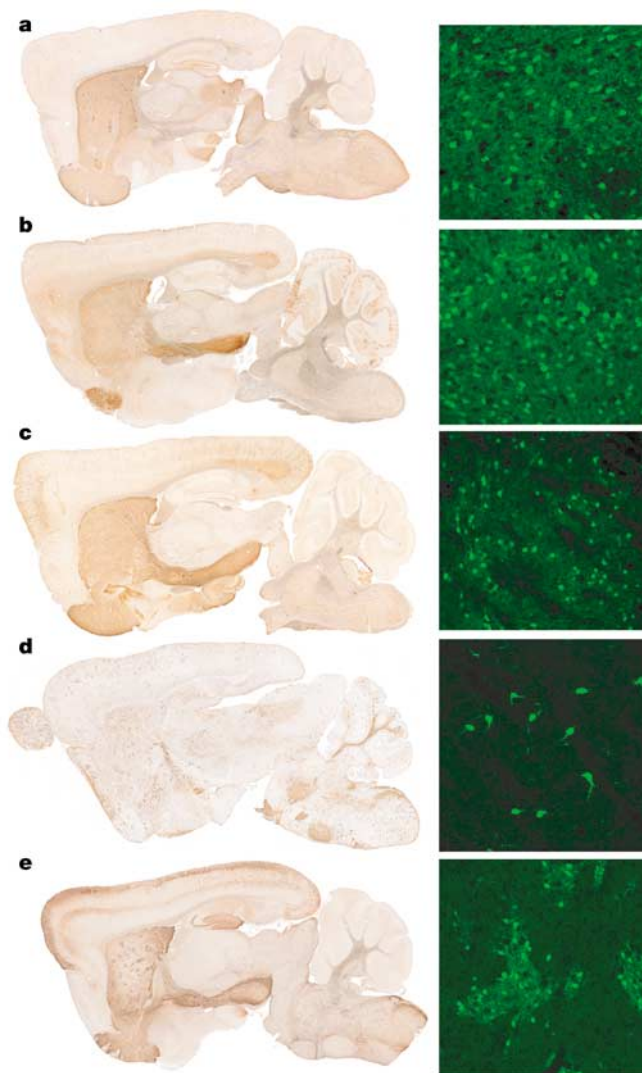


Figure 7 Cell-specific EGFP marker expression in the adult striatum of BAC transgenic mice. Sagittal sections of adult mice are shown immunostained with antibodies against EGFP (diaminobenzidine). **a**, In dopamine receptor *Drd2* BAC transgenic mice, the medium spiny neurons (right panel, confocal microscopy) are labelled. **b**, Dopamine receptor *Drd1a* transgenic mice. **c**, In *Chrm4* transgenic mice, the nigrostriatal pathway is stained and medium spiny neurons are labelled (right, confocal microscopy). **d**, In *Chat* BAC transgenic mice, cholinergic interneurons of the striatum are labelled. **e**, *Pdyn* expression in striatal patches.

targeted mouse lines requires significant effort, in our experience it is the detailed analysis of these lines and the presentation of the data to the public that is rate limiting.

Second, the ability to visualize individual cells using the EGFP reporter has revealed that a large number of the genes studied so far are expressed in novel subsets of classically defined cell types. It is apparent from these data that these subsets of cells can also share other important features; for example, they may all project to the same area of the CNS, or expression may mark distinct subpopulations in multiple CNS structures. We believe that, in many cases, these differences portend distinct functional properties, and anticipate that similar subdivisions of classical cell types will be observed as these methods are applied to other organ systems. This will require careful reconsideration of the meaning and definition of specific cell types *in vivo*.

Third, our experience so far has highlighted the advantages and limitations of the BAC reporter gene approach. The main advantages are the relative ease of producing BAC transgenic animals, the ability to identify lines carrying multiple copies of the BAC transgene in the genome, and the use of transgenic techniques in other vertebrate species. Several methods are now available for manipulation of BACs by homologous recombination^{3,4,41,42}, which can allow the construction of complex cassettes for *in vivo* manipulation of genes, cell types and tissues. A single day of pronuclear injection will normally yield multiple BAC transgenic founders (see Supplementary Information) that all transmit the transgene to subsequent generations. For these reasons, the use of well-characterized BAC vectors for *in vivo* manipulation can often proceed more efficiently than conventional gene targeting. Furthermore, anecdotal evidence from our laboratories and those of our colleagues points to many cases in which targeting EGFP into an endogenous genomic locus using homologous recombination ('knock in') has failed to produce sufficient reporter gene expression to be directly visualized in tissue slices or *in vivo*. This is consistent with the general observation reported here that, in many cases, EGFP fluorescence cannot be detected unless more than five copies of the BAC transgene are present in the genome. The ability to produce BAC transgenic lines expressing a wide range of dosages of the BAC transgene has many important applications for both vital imaging and genetic analysis. Finally, gene targeting is not yet feasible in many species that can be manipulated by pronuclear injection (cows, rats, zebrafish and so on). The main limitations to this approach to gene expression analysis are that it cannot be routinely used to study very large genes (>150 kb), and that it is considerably less efficient than lower-resolution techniques such as *in situ* hybridization. For this reason, the GENSAT project now includes a pre-screen using radioactive *in situ* hybridization to examine a large number of genes each year (T. Curran, unpublished data). From this pre-screen, 250–300 genes are chosen for in-depth analysis using BAC transgenic mice.

Fourth, the use of transgenic constructs as the basis for a gene expression screen provides valuable insights into eukaryotic gene regulation. Thus, the data obtained in using this approach identify individual BACs as either sufficient or insufficient to mediate correct gene expression *in vivo* (Fig. 1), and precisely identify the cell types that can be accessed using this segment of genomic DNA. This sort of experimental information is critical in trying to decipher the code for transcriptional regulation. It also identifies a starting point for dissection of transcriptional regulatory regions, and for the construction of artificial, small promoters to target gene expression *in vivo*.

Finally, the ability to specifically and reproducibly target expression to specific CNS cell populations using well-defined BAC vectors offers the opportunity to dramatically expand the capability for cell-specific genetic manipulation of the mouse genome. Thus, BAC transgenic mice expressing site-specific recombinases, increased levels of wild-type proteins, dominant-activating

or negative alleles, and other proteins and RNAs of interest in specific CNS cell types, have been or are being produced using BAC vectors identified in the GENSAT project.

Concluding remarks

The publication of the initial results of the GENSAT BAC Transgenic Project marks the beginning of a major effort to use genome mapping and sequence information in a directed, large-scale endeavour to advance mammalian neuroscience research. For the first time, the framework for completion of a high-resolution atlas of CNS gene expression, for the generation of a comprehensive library of BAC vectors to access defined CNS cell types, and for the creation of a repository of mice carrying labelled CNS cell populations has been established. Over the past 2 years, improvements in the methodology used in this screen, and in the availability and accuracy of the mouse genome databases, have markedly improved the efficiency of this project. Continued improvements in reporter gene technology, in the elements required for effective expression of polycistronic transcripts *in vivo*, and in inducible recombinases for genetic manipulation will further enhance the utility of this effort. We view the GENSAT BAC Transgenic Project as complementary to ongoing, large-scale directed and non-directed phenotypic screens to test CNS gene function. It is our hope that the examples we have discussed and the data we have released will accelerate research in all areas of neuroscience, and that access to the BAC vectors and transgenic lines we have produced will stimulate the fusion of mammalian molecular genetics and functional analysis of CNS cells and circuits. □

Methods

BAC transgene construction

The plasmid (pLD53.SC2) used in the homologous recombination is a derivative of pLD53 (ref. 43). pLD53 was digested with *Bam*HI and *Sac*I to get rid of the *tetAR* and *oriT* origin and replaced by *Not*I–*Sal*I–*Spe*I adaptor. A 1.1 kb EGFP.PA was cloned into *Not*I and *Sal*I sites, and an *Asc*I–*Not*I–*Swa*I–*Sma*I multiple cloning site was then cloned into the *Not*I site, which was knocked out thereafter. This shuttle vector (pLD53.SC2) was digested with *Asc*I/*Sma*I and purified by running on a 1% low-melting agarose gel. A 300–500 bp 'A Box' fragment used for the homologous recombination was amplified by polymerase chain reaction (PCR), digested with *Asc*I and cloned into this shuttle vector.

BAC host cells were streaked on an agar plate supplemented with chloramphenicol (Chl) (20 µg ml⁻¹) and incubated overnight at 37 °C. A single colony was picked, inoculated with 5 ml of Luria broth (LB) supplemented with Chl (20 µg ml⁻¹), and grown to optical density (OD) 600 of 0.5. The cells were harvested by centrifugation at 3,000 r.p.m. for 10 min at 4 °C. The pellet was suspended with 5 ml of ice-cold 50 mM CaCl₂, placed on ice for 5 min and harvested as above. The pellet was suspended with 300 µl of ice-cold solution of 50 mM CaCl₂ and 20% glycerol. An aliquot of 5 µl of PSV1.RecA plasmid (20 ng µl⁻¹) was transformed into 100 µl of competent cells; 1 ml of SOC was added, cells were incubated at 30 °C for 1.5 h with shaking at 225 r.p.m., and selected in 5 ml of LB containing tetracycline (Tet; 5 µg ml⁻¹) and Chl (20 µg ml⁻¹) at 30 °C overnight with shaking at 300 r.p.m. The overnight culture was diluted 1:50, inoculated with 50 ml of LB containing Tet (5 µg ml⁻¹) and Chl (20 µg ml⁻¹) in a 500 ml flask, and grown to OD 600 of 0.6–0.8 at 30 °C (4–5 h). The cells were harvested by centrifugation in a cold rotor at 3,000 r.p.m. for 10 min (in a Beckmann J6-MI centrifuge). The competent cells were prepared according to published protocols⁴. An aliquot of 2 µl of shuttle vector (500 ng µl⁻¹) was transformed into 40 µl of competent cells by electroporation. After the recovery, the cells were selected in 5 ml of LB containing Tet (5 µg ml⁻¹), ampicillin (Amp; 50 µg ml⁻¹) and Chl (20 µg ml⁻¹), and incubated at 30 °C overnight. The overnight culture (10 µl and 100 µl) was spread onto LB plates containing Amp (50 µg ml⁻¹) and Chl (20 µg ml⁻¹), and incubated overnight at 43 °C. Cointegrates were identified as reported previously⁴.

Modified BAC DNA was prepared by double acetate precipitation and CsCl gradient separation. The quality and concentration of the DNA were checked on pulse field gel. DNA was diluted to 2 ng µl⁻¹ and dialysed on a 25 mm, 0.025 µm filter (VSWP02500, Millipore) by floating it on 20 ml of injection buffer with the shiny side up for 3–4 h. An aliquot of DNA was removed to another tube and mixed with the same amount of ×2 polyamine (50:50) 48 h to 1 week before the injection. The BAC DNA (0.15–1 ng µl⁻¹) was injected into 200 pronuclei of fertilized oocytes of FVB/N mice.

Histology

Neonatal and adult animals were anaesthetized (Nembutal) and perfused with 4% paraformaldehyde. The brain and spinal cord were removed and post-fixed (4% paraformaldehyde, 1 h). Embryos were removed from deeply anaesthetized dams by laparoscopy and placed in ice-cold phosphate-buffered saline (PBS), and post-fixed by immersion in 4% paraformaldehyde (12 h, 4 °C). Serial sagittal sections (16 µm) were generated with a Micron or Leica cryostat, post-fixed in methanol (1 min, 4 °C) and

washed in PBS. Slides were either used directly for confocal imaging or immunostained using antibodies against EGFP (gift of M. Rout, Rockefeller University), horseradish peroxidase (using the TSA Biotin System, NEN) and ABC Vectastain (Vector Laboratories) methods. Primary antibody was added (1:2–10,000) overnight at 4 °C. For each neonatal and adult BAC transgenic animal, a set of 10–11 serial sections was generated and processed as above. Adult sections were chosen to match the series presented in the Paxinos atlas⁴⁴, with sections 1–10 matching Paxinos sections 129, 126, 123, 120, 117, 114, 111, 108, 105 and 102, respectively. Transverse spinal cord sections were C4–C6, T3–T6 and L6–S1. P7 sections were matched to sections from the Valverde atlas⁴⁵, with sections 1–11 matching Valverde figures 25–35, respectively. Transverse spinal cord sections were C4–C6, T3–T6 and L6–S1. E15.5 sections were chosen to match the Schambra atlas⁴⁶ with sections 1–10 matching Schambra figures 1–10, respectively. Transverse spinal cord sections were C4–C6, T3–T6 and L6–S1. For E10.5 embryos, the maximum number of sections possible was generated.

Images were acquired with a Zeiss Axiocam camera on a Zeiss Axioskop2 microscope with a ×5 and ×10 (1.5 numerical aperture) objective and an automated x,y stage (Marzhauser scan8) controlled and hosted by a PC with Zeiss KS400 software running a macro written by us. Adult sagittal sections were approximately 200 MB. The final resolution of the ×10 images was approximately 1.33 μm per pixel. Confocal images were acquired with a Zeiss Axioskop2 using a BioRad Radiance confocal imaging system.

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Motor neuron columnar fate imposed by sequential phases of Hox-c activity

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The organization of neurons into columns is a prominent feature of central nervous system structure and function. In many regions of the central nervous system the grouping of neurons into columns links cell-body position to axonal trajectory, thus contributing to the establishment of topographic neural maps. This link is prominent in the developing spinal cord, where columnar sets of motor neurons innervate distinct targets in the periphery. We show here that sequential phases of Hox-c protein expression and activity control the columnar differentiation of spinal motor neurons. Hox expression in neural progenitors is established by graded fibroblast growth factor signalling and translated into a distinct motor neuron Hox pattern. Motor neuron columnar fate then emerges through cell autonomous repressor and activator functions of Hox proteins. Hox proteins also direct the expression of genes that establish motor topographic projections, thus implicating Hox proteins as critical determinants of spinal motor neuron identity and organization.

Columns are basic units of neuronal organization in the vertebrate central nervous system (CNS)^{1,2}, but the mechanisms that control their assembly remain unclear. One region of the CNS in which neuronal columnar organization has been studied intensively is the spinal cord, where distinct columnar subtypes of motor neurons are known to innervate different muscle and neuronal targets in the periphery³. All spinal motor neurons (MNs) derive from progenitor cells located at a constant dorso-ventral position in the neural tube⁴, but motor neurons appear to acquire their discrete columnar identities as a function of position along the rostrocaudal axis of the spinal cord^{5–8} (Supplementary Fig. S1a–c). Limb levels of the spinal cord generate lateral motor column (LMC) neurons, whereas intervening thoracic levels give rise to autonomic MNs (termed Column of Terni (CT) neurons in chick)^{5–8}. Along the dorsoventral axis, the graded signalling activity of Sonic hedgehog (Shh) determines the position of MN generation by regulating the spatial expression of a set of homeodomain transcription factors within neural progenitors^{4,9}. The constancy of Shh signalling along the rostrocaudal axis, however, implies that other signalling pathways control the diversification of MNs into distinct columnar subtypes.

One source of signals that can influence the columnar fate of spinal motor neurons is the paraxial mesoderm. The transposition of paraxial mesoderm between limb and thoracic levels soon after neural tube closure results in the respecification of LMC and CT identity, and a similar switch in columnar fates is observed after transposition of the neural tube¹⁰. However, before neural tube closure, signals from axial mesodermal tissues—the node and nascent notochord—have been shown to regulate the molecular identity of MNs along the rostrocaudal axis of the spinal cord¹¹. In particular, members of the Hox-c homeodomain protein cluster are expressed by post-mitotic MNs generated at distinct rostrocaudal positions¹¹, and profiles of Hox-c protein expression characteristic of brachial, thoracic and lumbar MNs can be induced *in vitro* by early exposure of neural cells to increasing levels of fibroblast growth factor (FGF) signalling^{11,12}. Moreover, the pattern of Hox-c protein expression changes along with columnar identity after early transposition of brachial and thoracic neural tube¹⁰. Hox proteins influence MN diversification in the hindbrain^{13–15}, and thus a role for Hox proteins in the assignment of spinal MN columnar identity is plausible. Nevertheless, the link between axial FGF signalling,

neural Hox protein expression, and the establishment of spinal MN columnar identity, if any, remains obscure.

Hox protein expression segregates with MN columnar subtype

To explore the link between Hox expression and MN columnar identity, we focused initially on Hox-c proteins, because members of this Hox cluster are prominently expressed by MNs¹¹. We examined how the expression of Hoxc5, Hoxc6, Hoxc8 and Hoxc9 at brachial and thoracic levels of the spinal cord is matched to the differentiation of LMC and CT neurons^{5,16}.

Hoxc6 expression by MNs (defined by Isl1/2 expression) was confined to brachial levels, whereas Hoxc9 expression by MNs was restricted to thoracic levels (Fig. 1a). Hoxc6⁺ and Hoxc9⁺ MNs were interspersed at the border of the brachial and thoracic spinal cord, but very few neurons expressed both proteins (Fig. 1a, g). The rostrocaudal domains of expression of Hoxc5 and Hoxc8 by MNs were also segregated (Fig. 1b, h), but in contrast to Hoxc6 and Hoxc9, the transition from Hoxc5⁺ to Hoxc8⁺ MNs mapped to the mid-brachial level (Fig. 1c; Supplementary Fig. S2c). Thus, rostral brachial LMC neurons co-express Hoxc5 and Hoxc6, whereas caudal brachial LMC neurons co-express Hoxc6 and Hoxc8 (Fig. 1c, e, f, i, k). At rostral thoracic levels, many MNs initially co-expressed Hoxc8 and Hoxc9 (Fig. 1d, j).

MN columnar subtypes located at different rostrocaudal positions can be delineated by LIM homeodomain transcription factor expression¹⁷. In addition, CT neurons can be distinguished by expression of *BMP5*, a transforming growth factor- β family member¹⁸, and LMC neurons by expression of retinaldehyde dehydrogenase-2 (*RALDH2*), a key enzyme in retinoic acid synthesis^{19,20} (Supplementary Fig. S1d). We therefore examined whether the profile of Hox-c protein expression by MNs corresponds to the position of newly generated brachial LMC neurons and of CT neurons. At brachial levels, the rostral and caudal boundaries of *RALDH2* expression coincided closely with the domain of Hoxc6⁺, Hoxc9[–] MNs (Fig. 1l, m, o; Supplementary Fig. S2a, b). At thoracic levels, the rostral and caudal boundaries of *BMP5* expression coincided with the domain of Hoxc9⁺, Hoxc6[–] MNs (Fig. 1n–p; Supplementary Fig. S2d, e). Together, these data indicate that Hoxc6 and Hoxc9 expression within MNs coincides with brachial LMC and thoracic CT columns, respectively (Fig. 1q).

Regulation of Hox-c protein expression by FGF signalling

The exposure of neural progenitor cells *in vitro* to increasing FGF levels induces the differentiation of MNs with a progressively more caudal Hox-c profile, suggesting a model in which the positional identity of MNs at brachial and thoracic levels is established by graded FGF signalling¹¹.

To test this model *in vivo*, we examined whether an increase in the level of FGF signalling in the neural tube elicits a rostral to caudal switch in the profile of Hox-c protein expression. *In ovo* electroporation was used to co-express *FGF8* and the green fluorescent protein gene *GFP* (as an indicator of transfected cells) unilaterally in brachial and thoracic regions of the stage 12 neural tube (Fig. 2a). The concentration of *FGF8* plasmid was titrated to a level that did not perturb neural tube morphology or change the dorsoventral pattern of progenitor homeodomain proteins (see Methods). Expression of *FGF8* resulted in the extinction of Hoxc6 expression and the onset of Hoxc9 expression at brachial levels (Fig. 2b–f). Similarly, exposure of rostral brachial levels to *FGF8* led to the extinction of Hoxc5 and to a rostral expansion in Hoxc8 expression (Fig. 2g, h). Thus, brachial FGF expression results in a brachial to thoracic switch in the profile of Hox-c expression.

Because profiles of Hox-c protein expression characteristic of lumbar levels of the spinal cord can be induced by early exposure of neural cells to high level FGF signalling^{11,12}, we also examined whether the expression of *FGF8* at thoracic levels resulted in a switch to a lumbar Hox-c expression profile. However, *FGF8* expression did not markedly influence the profile Hox-c expression at thoracic levels (Supplementary Fig. S3a–e), a finding likely to reflect a comparatively low level of FGF8 signalling achieved under these electroporation conditions.

FGF signalling and the specification of MN columnar identity

We next examined whether increased FGF8 signalling in brachial

neural tube influences MN columnar identity. Brachial expression of *FGF8* did not suppress the generation of Isl1/2⁺ MNs, but reduced the total MN number by ~30% at stage 29 (Fig. 2i, j), a finding consistent with observations that the number of MNs generated at brachial levels of the spinal cord normally exceeds that at thoracic levels²⁰. The number of medial MMC neurons, assessed by Lim3 expression¹⁷ was not altered (Supplementary Fig. S3i), consistent with the presence of this motor column at both brachial and thoracic levels.

After *FGF8* expression, however, brachial MNs lacked RALDH2 expression (Fig. 2k). The expression of RALDH2 by LMC neurons directs lateral LMC divisional identity, defined by co-expression of Isl2 and Lim1¹⁷. Consistent with this, we detected a virtually complete loss of Isl2⁺, Lim1⁺ MNs (Fig. 2j). Thus, increased FGF8 signalling in brachial neural tube suppresses LMC differentiation in a manner that parallels the suppression of Hoxc6 expression.

We examined whether the loss of LMC columnar identity is accompanied by the emergence of a thoracic programme of MN columnar differentiation. After brachial *FGF8* expression and analysis at stage 29, the characteristic ovoid clustering of MNs typical of the LMC was lost, and instead MNs were clustered in a semilunar arrangement, typical of thoracic MNs¹⁷ (Fig. 2j). Moreover, many brachial MNs now expressed the bone morphogenetic protein gene *BMP5* (Fig. 2l), indicating that they have acquired a CT identity. Analysis at stage 29 revealed that many islet-1⁺ (Isl1⁺), *BMP5*⁺ MNs had migrated to a dorsomedial position typical of CT neurons (Fig. 2j, Supplementary Fig. S3j). Thus, elevated brachial FGF8 signalling results in a conversion from LMC to CT columnar identity, paralleling the switch from Hoxc6 to Hoxc9 expression.

Several lines of evidence indicate that the brachial to thoracic switch in Hox-c expression and MN columnar identity results from a direct action of FGF8 on neural cells. Brachial neural expression of

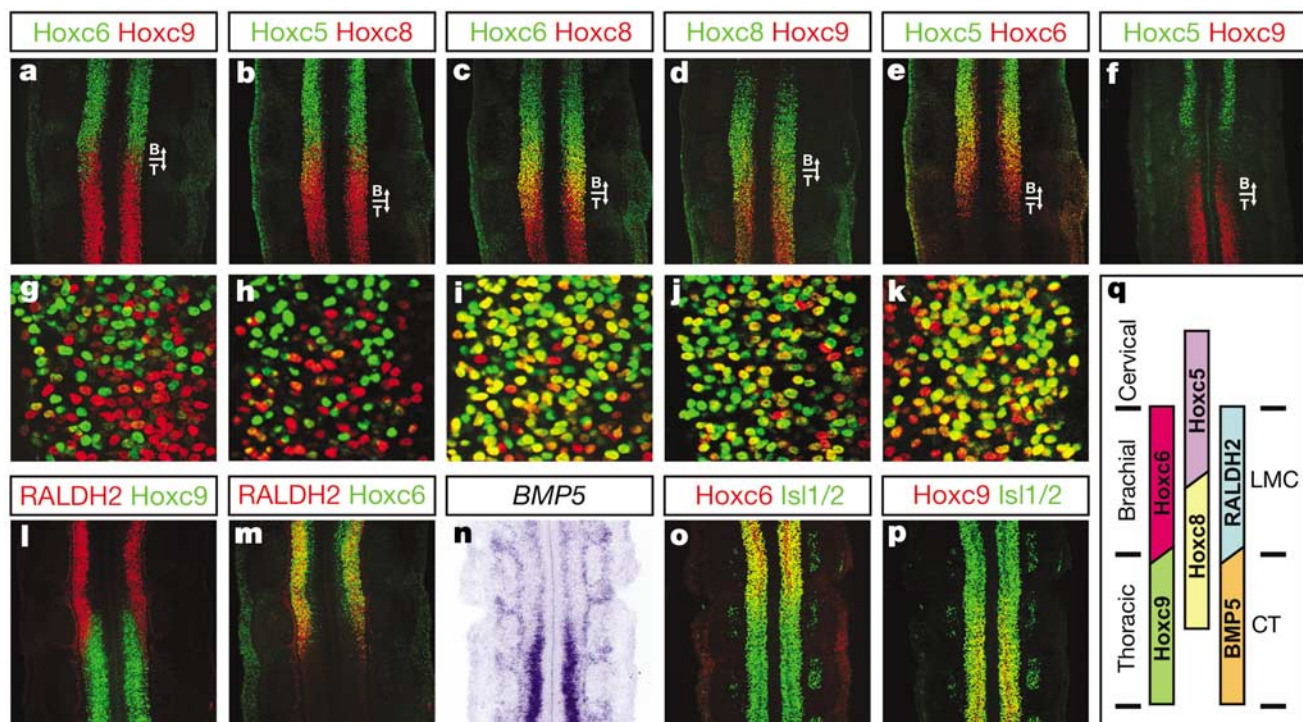


Figure 1 Hox-c protein expression and spinal motor neuron columnar identity. **a–f**, Hoxc5, Hoxc6, Hoxc8 and Hoxc9 protein expression in horizontal longitudinal sections of stage 24 brachial and thoracic chick spinal cord. T-junction indicates position of the brachial (B) and thoracic (T) border. **g–k**, Higher magnification images of Hox-c expression reveals few if any Hoxc6⁺/Hoxc9⁺ or Hoxc5⁺/Hoxc8⁺ neurons, but many

Hoxc6⁺/Hoxc8⁺, Hoxc8⁺/Hoxc9⁺, and Hoxc5⁺/Hoxc6⁺ neurons. **l–p**, Domains of Hoxc6 and Hoxc9 expression in stage 24 chick spinal cord, in comparison to RALDH2, *BMP5* and Isl1/2 expression. **q**, Rostral-caudal domains of Hoxc5, Hoxc6, Hoxc8 and Hoxc9 expression in MNs, with respect to molecularly defined MN columns.

a constitutively active FGF type I receptor that functions in a cell-autonomous manner^{11,21} mimicked the actions of FGF8 in suppressing Hoxc6 and RALDH2 expression, and in inducing Hoxc9 and BMP5 expression¹¹ (Supplementary Fig. S4a–d). In addition, expression of FGF8 in brachial neural cells did not change the Hox-c profile of the adjacent paraxial mesoderm (data not shown). Moreover, the spatial extent of FGF8 signalling, revealed by di-phosphorylated mitogen-activated protein kinase (MAPK) (ERK1/2) expression²², appeared to be confined to neural cells (Supplementary Fig. S4e–g).

Specification of MN columnar identity by Hox-c proteins

If the FGF8-elicited switch from LMC to CT columnar fate is mediated by changes in Hox expression, then ectopic expression of individual Hox-c proteins might be expected to change MN columnar fate.

To test this idea, we expressed Hoxc9 in stage 12 brachial neural tube, using a CMV/ β -actin promoter (pCAGGS), and analysed changes in Hox-c expression and MN columnar identity at stages 27 to 29. Brachial expression of Hoxc9 inhibited Hoxc6 expression by MNs (Fig. 3a; <3% of MNs co-expressed Hoxc9 and Hoxc6). Moreover, the suppression of Hoxc6 expression was restricted to MNs that expressed Hoxc9 (Fig. 3a), indicating the cell-autonomy of Hoxc9 action. Brachial expression of Hoxc9 suppressed LMC differentiation in a cell-autonomous manner, as assessed by the loss

of RALDH2 expression (Fig. 3b, c), and also markedly reduced the number of Isl2⁺, Lim1⁺ lateral LMC neurons (Fig. 3d). Remarkably, many brachial Hoxc9⁺, Isl1/2⁺ MNs expressed BMP5 (Fig. 3f), and by stage 29, many Isl1⁺, Hoxc9⁺, BMP5⁺ MNs were located in a dorsomedial position, characteristic of CT neurons (Fig. 3e, g). Thus, brachial expression of Hoxc9 mimics the effects of increased FGF8 signalling, repressing Hoxc6 expression and LMC differentiation, and inducing CT identity.

We next examined whether thoracic Hoxc6 expression elicits a caudal to rostral switch in MN columnar identity. Thoracic Hoxc6 misexpression led to the cell-autonomous repression of Hoxc9 expression by MNs, prevented expression of BMP5, and inhibited the later dorsomedial migration of Isl1⁺ MNs (Fig. 3h–j and data not shown). In addition, many thoracic Hoxc6⁺ MNs acquired an LMC identity, assessed by RALDH2 expression, the presence of Isl2⁺, Lim1⁺ lateral LMC neurons, and by expression of Nkx6 proteins, additional markers of LMC neurons²³ (Fig. 3k–o). Forced expression of Hoxc6 or Hoxc9 within their normal segmental domains did not change the profile of MN columnar differentiation (data not shown). Together, these findings provide evidence that Hoxc6 and Hoxc9 specify, respectively, brachial LMC and thoracic CT identity. The corresponding Hox-a paralogues, Hoxa6 and Hoxa9, have similar expression patterns and MN columnar specification activities (Supplementary Figs S5a–d and S6a–e).

The activities of Hox6 and Hox9 proteins also appear selective.

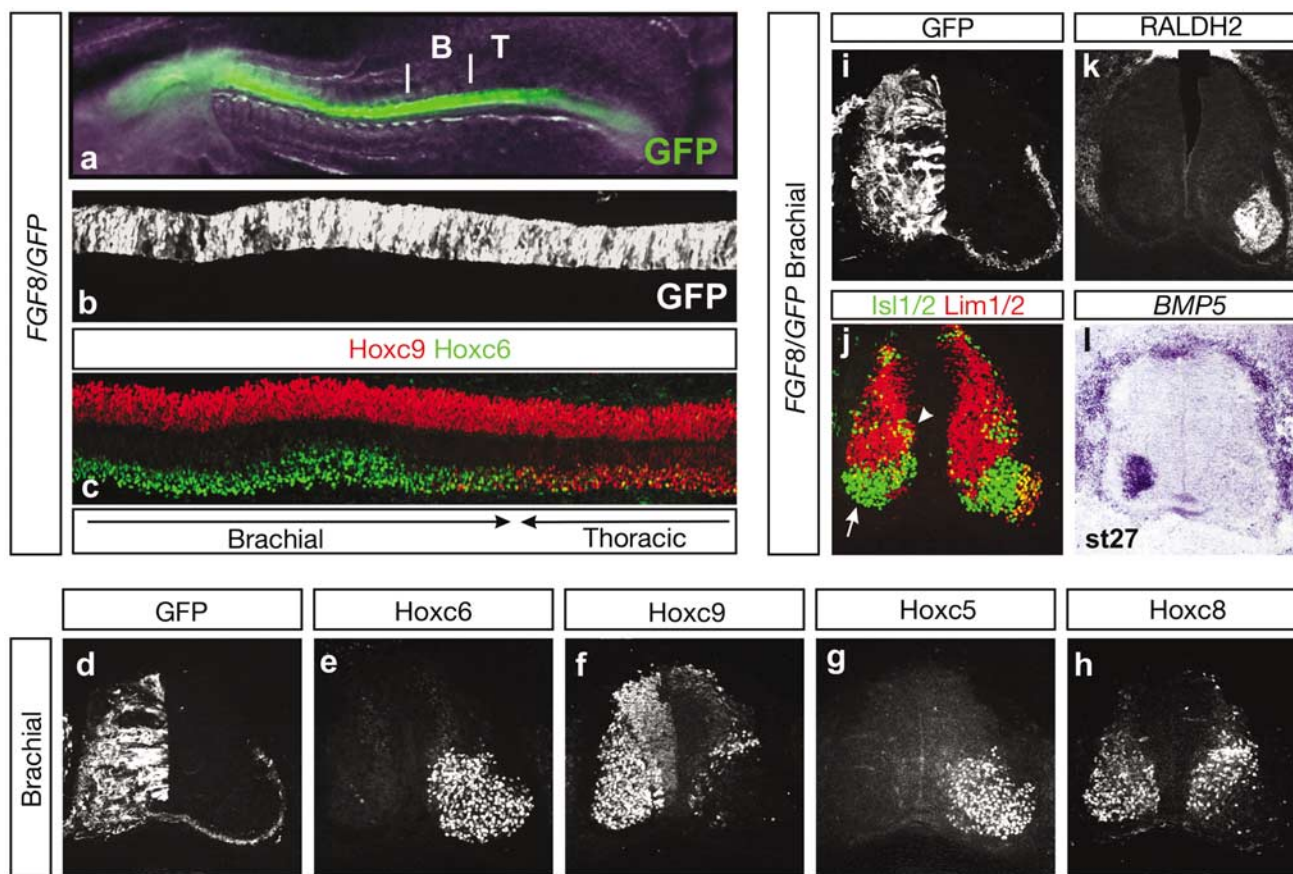


Figure 2 Hox-c and motor column repatterning after FGF8 expression in brachial neural tube. **a**, Top-down image of a chick embryo 8 h after unilateral co-electroporation of CMV-enhanced GFP and CMV-FGF8 expression plasmids at stage 12. GFP is expressed at brachial (B) and thoracic (T) levels. **b, c**, Top-down views of brachial and thoracic levels of a chick spinal cord, ~48 h after FGF8/GFP electroporation stage 12. **d–h**, Hox-c expression in cross-sections of stage 29 brachial spinal cord after FGF8/GFP electroporation. Ectopic expression of Hox-c proteins is detected in MNs and ventral

interneurons. Loss of Hoxc6 protein is accompanied by loss of Hoxc6 mRNA (see Supplementary Fig. S3k, l). **i–l**, MN columnar marker expression after brachial FGF8/GFP electroporation. The loss of lateral LMC neurons (arrow in Fig. 2j) is accompanied by the appearance of dorsomedially positioned Isl1⁺ MNs (arrowhead in Fig. 2j). **l**, BMP5 expression at stage 27 after brachial FGF8 electroporation. The differentiation of MN columnar subtypes was not markedly affected at thoracic levels of the spinal cord (Supplementary Fig. S3e–h).

Expression of *Hoxc6* at rostral thoracic levels failed to suppress *Hoxc8* and conversely, ectopic expression of *Hoxc8* at rostral brachial levels did not suppress *Hoxc6* or *RALDH2* expression (Supplementary Fig. S6f–i). In contrast, expression of *Hoxc8* at rostral brachial levels suppressed *Hoxc5* expression (Supplementary Fig. S6j), providing evidence that the exclusive domains of expression of *Hoxc5* and *Hoxc8* within the LMC are also established by selective cross-repressive interactions.

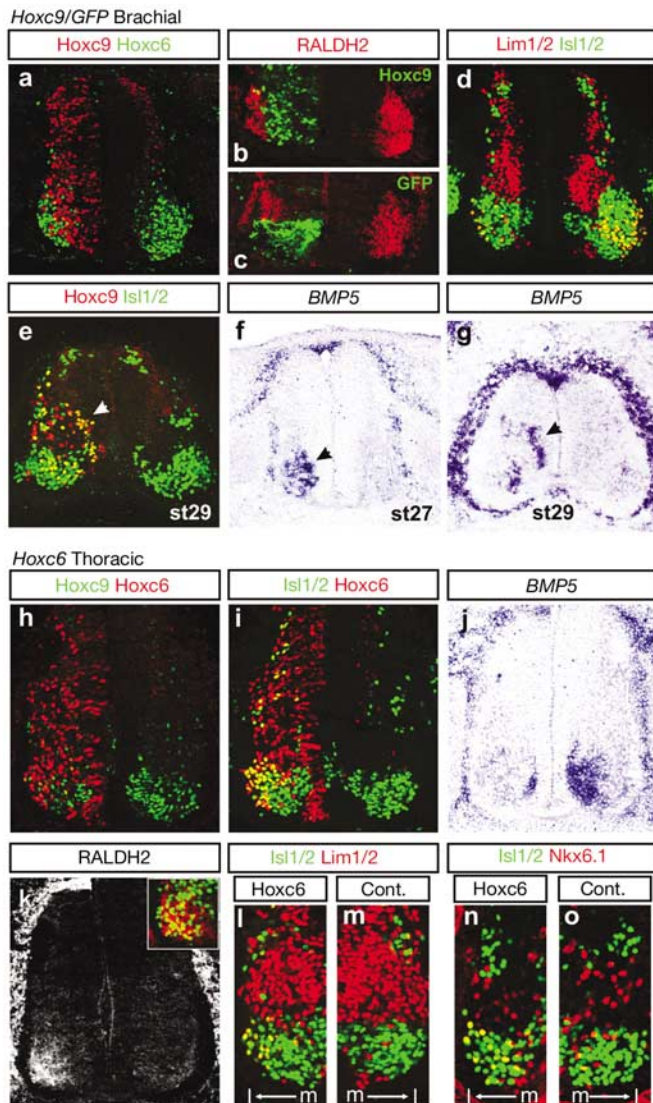


Figure 3 Motor columnar patterning activities of *Hoxc9* and *Hoxc6*. **a**, Pattern of *Hoxc6* after expression of chick *Hoxc9* in stage 12 brachial neural tube, and analysis at stage 27. **b–d**, Pattern of *RALDH2* expression and reduction in the number of *Isl2*⁺, *Lim1*⁺ lateral LMC MNs after brachial expression of *Hoxc9* *GFP*. **e**, Dorsomedial position of *Hoxc9*⁺, *Isl1*⁺ MNs (white arrowhead) after *Hoxc9* expression. **f, g**, Induction of *BMP5* expression in premigratory (stage 27) and dorsomedially positioned (stage 29) MNs (black arrowhead). **h**, Expression of *Hoxc9* after thoracic expression of murine *Hoxc6* in stage 12 neural tube. **i**, *Hoxc6*⁺/*Isl1*⁺ MNs settle in a ventrolateral position. **j**, *BMP5* expression after thoracic *Hoxc6* expression. **k**, Induction of *RALDH2* expression in *Hoxc6*⁺ MNs. Inset shows co-expression of *Hoxc6* (green) and *RALDH2* (red). **l–o**, Expression of LMC markers after thoracic *Hoxc6* expression. Many *Isl1/2*⁺ MNs co-express *Lim1* (**l, m**) and *Nkx6.1* (**n, o**). Medial (m) and lateral (l) positions in the spinal cord are indicated. Medial MMC neurons at brachial and thoracic levels express *Hoxc6* and *Hoxc9*, respectively. At both levels some MNs retain a medial MMC identity, assessed by LIM homeodomain protein *Lim3* expression, after expression of the segmentally inappropriate Hox-c protein (data not shown). Cont., contralateral side.

Hox6 and Hox9 expression and regulation in neural progenitors

Does the profile of Hox expression in post-mitotic MNs emerge from an earlier rostrocaudal difference in *Hox6* and *Hox9* expression by neural progenitors? Expression of *Hox6* paralogues was absent from brachial progenitor cells between stages 10 to 14, whereas expression of *Hoxc6* messenger RNA was detected in thoracic progenitors (Supplementary Fig. S5e–g). *Hoxc6* protein, however, was not detected in thoracic progenitors (Fig. 4f). Expression of *Hoxa9*, *Hoxb9* and *Hoxc9* mRNA and *Hoxc9* protein was detected in thoracic neural progenitors at stage 15, just before the generation of post-mitotic MNs (Fig. 4a–c, e). Each *Hox9* gene was expressed over the same general domain, with a rostral limit of expression at somites 20/21 (Fig. 4a–c). Taken together, these findings indicate that brachial progenitors lack expression of *Hox6* and *Hox9* proteins, whereas thoracic progenitors express *Hox9* but not *Hox6* proteins (Fig. 4j).

We next examined whether FGF8 signalling changes the profile of *Hox* gene expression in neural progenitors. After brachial *FGF8*

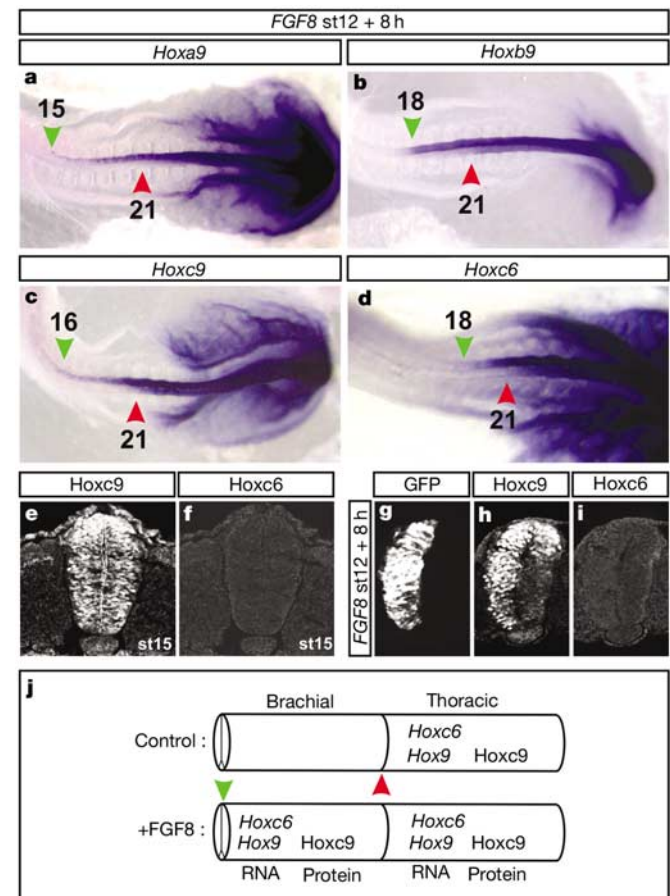


Figure 4 Rapid repatterning of neural progenitor Hox profile after brachial *FGF8* expression. **a–d**, Rostral expansion in domains of expression of *Hox9* paralogues and of *Hoxc6*, 8 h after unilateral *FGF8* electroporation. Red arrowhead indicates normal rostral expression boundary, green arrowhead indicates rostral boundary after exposure to *FGF8*. **e, f**, Expression of *Hoxc9* and absence of *Hoxc6* protein in stage 15 thoracic neural tube. **g–i**, Ectopic brachial expression of *Hoxc9* protein, but not *Hoxc6* protein, 8 h after *FGF8* electroporation in stage 12 neural tube. The restriction of the repressive activity of Hox9 proteins to post-mitotic neurons could reflect gating of Hox activities by co-factors such as Meis/Pbx proteins⁴⁶, a possibility supported by the restricted expression of Meis1 and Pbx3 in MNs (J.S.D and T.M.J., unpublished observations). **j**, Schematic of *Hox6* and *Hox9* mRNA and protein in brachial and thoracic neural tube in normal and *FGF8*-exposed embryos. A mismatch in *Hoxc6* mRNA and protein has also been reported in the chick limb⁴⁷, suggesting a more general constraint on *Hoxc6* expression. By analogy with other Hox genes⁴⁸, this regulation may involve post-transcriptional regulatory controls.

expression at stage 12 and analysis 8 h later, ectopic rostral expression of *Hoxa9*, *Hoxb9*, and *Hoxc9* mRNAs and *Hoxc9* protein was detected within neural progenitors (Fig. 4a–c, g, h). Expression of *FGF8* also resulted in a rostral expansion in the domain of *Hoxc6* mRNA expression (Fig. 4d), although *Hoxc6* protein was still not detected (Fig. 4i). The pattern of *Hoxc6* and *Hox9* expression in thoracic progenitors was not markedly altered by *FGF8* expression (Fig. 4a–c). Moreover, expression of *FGF8* at stage 17, after the onset of MN differentiation, did not alter the profile of Hox-c expression (Supplementary Fig. S4h–k). Together, these findings indicate that exposure of brachial progenitors to *FGF8* rapidly induces *Hoxc9* expression, in the absence of *Hoxc6* protein, thus establishing a Hox profile characteristic of thoracic level progenitors (Fig. 4j).

Hox-c activities in post-mitotic MNs

The difference in the time of onset of *Hox6* and *Hox9* protein expression at brachial and thoracic levels led us to examine when the activity of these two Hox paralogue groups is required for MN columnar specification. To address this issue, we examined whether MN columnar identity can be respecified by selective expression of Hox proteins in post-mitotic MNs. To restrict Hox protein expression, we used a 9-kilobase (kb) 5' flanking region of the mouse *Hb9* gene, which confers expression to post-mitotic neurons and excludes expression from progenitor cells^{18,24}.

Expression of *Hoxc9* in post-mitotic brachial MNs suppressed *Hoxc6* expression and blocked the differentiation of *RALDH2*⁺ LMC neurons, and of *Isl2*⁺, *Lim1*⁺ lateral LMC neurons (Fig. 5a–c and data not shown). However, post-mitotic *Hoxc9* expression failed to induce CT differentiation, as revealed by the lack of *BMP5* expression,

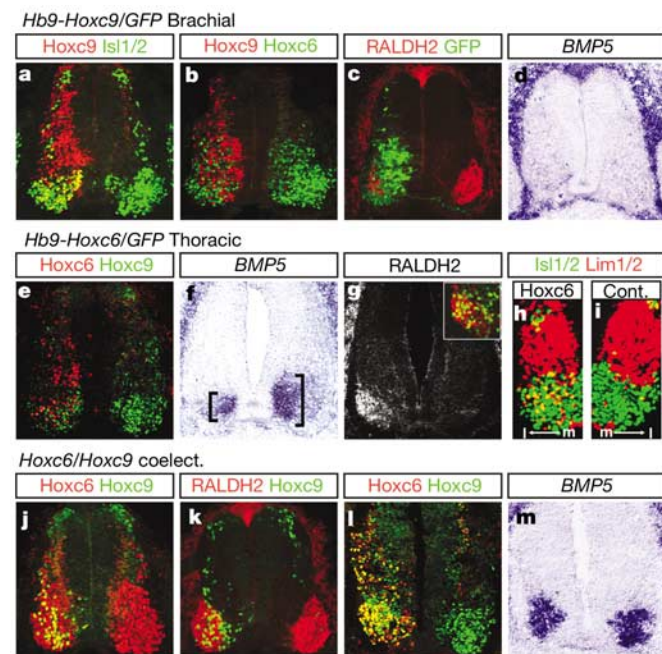


Figure 5 Activities of *Hoxc6* and *Hoxc9* in post-mitotic neurons. **a**, Expression of *Hb9-Hoxc9/GFP* at brachial levels results in many neurons that co-express *Isl1/2* and *Hoxc9*. Even though the *Hb9* promoter occasionally results in ectopic Hox expression in interneurons as well as MNs, no expression is detected in progenitor cells. **b, c**, Loss of *Hoxc6* and *RALDH2* expression after brachial post-mitotic expression of *Hoxc9*. **d**, Expression of *Hoxc9* in brachial MNs does not induce *BMP5* expression. **e, f**, Repression of *Hoxc9* and *BMP5* expression after post-mitotic thoracic expression of *Hoxc6*. **g–i**, Expression of *Hoxc6* in thoracic MNs induces expression of *RALDH2* and co-expression of *Isl2* and *Lim1*. Inset in **g** indicates co-expression of *Hoxc6* (green) and *RALDH2* (red). **j–m**, Effect of *Hoxc6* and *Hoxc9* co-expression at brachial (**j, k**) and thoracic (**l, m**) levels.

and of dorsomedially located *Hoxc9*⁺, *Isl1*⁺ MNs (Fig. 5a, d and data not shown). These findings provide evidence that *Hoxc9* function is required in brachial progenitors as well as post-mitotic MNs for the ectopic specification of CT identity.

Conversely, expression of *Hoxc6* in post-mitotic thoracic MNs repressed *Hoxc9* expression and prevented the expression of *BMP5* by thoracic MNs (Fig. 5e, f). But in addition, post-mitotic *Hoxc6* expression induced expression of *RALDH2*⁺ LMC neurons, and *Isl2*⁺, *Lim1*⁺ lateral LMC neurons (Fig. 5g–i). Thus, *Hoxc6* can specify brachial LMC identity solely through actions in post-mitotic MNs.

To examine whether the post-mitotic blockade of LMC differentiation by *Hoxc9*, and of CT differentiation by *Hoxc6*, depends primarily on repression of the complementary Hox-c protein, we assayed the consequences of forced co-expression of *Hoxc6* and *Hoxc9* at brachial and thoracic levels. At brachial levels, many MNs that expressed *Hoxc9* also expressed *RALDH2* (Fig. 5j, k), and at thoracic levels *BMP5* expression was preserved, despite widespread expression of *Hoxc6* (Fig. 5l, m). These findings provide evidence that the ability of *Hoxc9* to block LMC differentiation, and of *Hoxc6* to block CT differentiation, is mediated primarily through repression of their complementary Hox proteins.

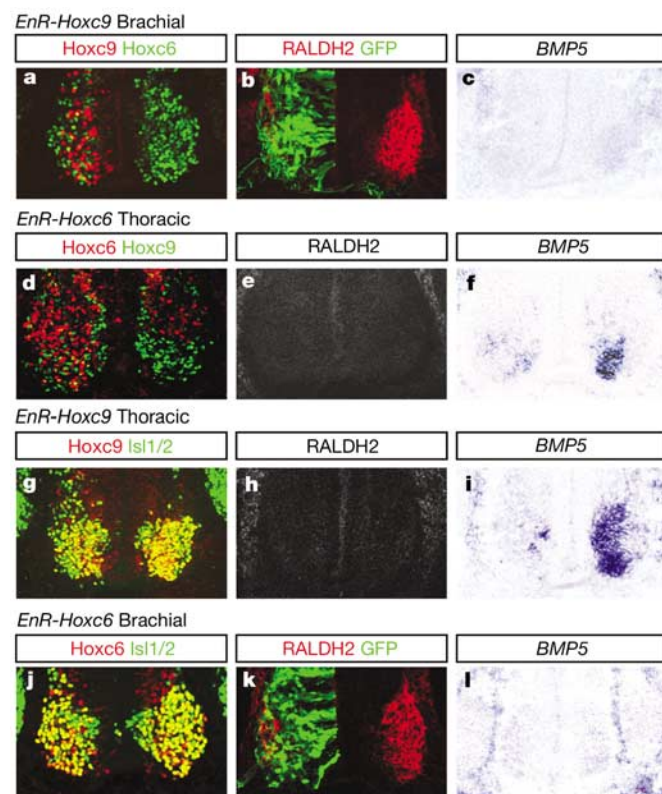


Figure 6 Actions of Engrailed repressor derivatives of *Hoxc6* and *Hoxc9* on columnar differentiation. **a–c**, Expression of *EnR-Hoxc9/GFP* in brachial neural tube represses *Hoxc6* and *RALDH2* expression, but fails to induce *BMP5* expression. **d–f**, Expression of *EnR-Hoxc6/GFP* at thoracic levels of the neural tube represses *Hoxc9* and *BMP5* expression, but fails to induce expression of *RALDH2*. We detected an increase in the proportion of MNs that expressed *Lim3* (data not shown), however, raising the possibility that some MNs have acquired a medial MMC-like character. **g–i**, Expression of *EnR-Hoxc9/GFP* in thoracic neural tube permits the generation of *Hoxc9*⁺, *Isl1/2*⁺ MN, but these neurons do not express *BMP5* or *RALDH2*. **j–l**, Expression of *EnR-Hoxc6/GFP* in brachial neural tube permits the generation of *Hoxc6*⁺, *Isl1/2*⁺ MNs, but these neurons do not express *BMP5* or *RALDH2*. Expression of *VP16-Hoxc6* and *VP16-Hoxc9* fusion proteins appear to function in a manner similar to their wild-type counterparts, a finding that parallels observations of Hox activity in *Drosophila*¹⁹.

Requirement for Hox-c activity in MN columnar specification

Hox proteins appear to specify MN columnar identity through mutual cross-repressive interactions as well as the induction of columnar differentiation markers, posing the question of whether Hox proteins function as repressors and/or activators. To address this issue, we generated presumed repressor derivatives of Hoxc6 and Hoxc9 by fusing full-length Hox-c coding regions to the repressor domain (EnR) of the *Drosophila* Engrailed protein²⁵. Expression of *EnR-Hoxc9* under *pCAGGS* control in the stage 12 brachial neural tube suppressed Hoxc6 expression and the differentiation of RALDH2⁺ LMC neurons, but failed to induce *BMP5* expression in MNs (Fig. 6a–c). Conversely, thoracic expression of *EnR-Hoxc6* under *pCAGGS* control suppressed Hoxc9 expression and the differentiation of *BMP5*⁺ CT neurons, but failed to induce RALDH2 expression (Fig. 6d–f). As a specificity control, expression of *EnR-Hoxc8* at brachial levels repressed Hoxc5 at rostral brachial levels but did not repress Hoxc6 expression or the differentiation of LMC neurons (Supplementary Fig. S7a–c). These findings suggest that the cross-repressive interactions of Hoxc9 and Hoxc6 reflect transcriptional repressor activities, whereas Hox activator functions are required for ectopic induction of columnar differentiation markers.

The apparent requirement for Hox activator function in columnar differentiation raises the issue of whether the EnR Hox-c derivatives inhibit LMC and CT differentiation within the normal domains of Hoxc6 and Hoxc9 expression. Thoracic expression of *EnR-Hoxc9* blocked the expression of *BMP5* in thoracic MNs, and prevented the later dorsomedial migration of MNs (Fig. 6g–i and data not shown). Conversely, brachial expression of *EnR-Hoxc6* blocked the differentiation of RALDH2⁺ LMC neurons (Fig. 6j–l). Thus, the loss of both LMC and CT columnar identity after *EnR-Hoxc6* or

EnR-Hoxc9 expression leaves most brachial and thoracic Isl1/2⁺ MNs without an appropriate columnar character, although their precise subtype identity remains to be established. Together, these data provide evidence that Hox6 and Hox9 activities are normally required for the specification of LMC and CT columnar identities at brachial and thoracic levels of the spinal cord.

Discussion

Hox function and MN columnar specification

Our findings reveal that the specification of MN columnar subtypes along the rostrocaudal axis of the spinal cord involves sequential phases of Hox expression and activity in progenitor cells and post-mitotic MNs (Fig. 7a). The positional identity of progenitor cells at brachial and thoracic levels is reflected in the status of Hox9 expression: brachial progenitors lack Hox9 protein expression, whereas thoracic progenitors express Hox9 proteins. Expression of Hox9 proteins by thoracic progenitors anticipates the expression of Hox9 proteins by thoracic MNs, and the absence of Hox9 protein expression provides a brachial progenitor context that permits the onset of Hox6 protein expression in post-mitotic brachial MNs. Nevertheless, our findings do not exclude the possibility that other Hox proteins contribute to brachial progenitor identity. The differentiation of brachial LMC neurons has been shown to depend on retinoid signals provided by the paraxial mesoderm^{10,26}. Thus, Hoxc6 expression and brachial LMC differentiation may depend both on an early progenitor Hox profile, and on a post-mitotic phase of retinoid signalling from the paraxial mesoderm.

The profile of Hox protein expression by post-mitotic MNs appears to be a major determinant of their columnar fate (Fig. 7a). Post-mitotic brachial misexpression of Hoxc9 inhibits LMC identity but is insufficient to direct CT identity, suggesting that sequential phases of Hox9 expression by thoracic progenitors and post-mitotic MNs are necessary for CT identity. In contrast, Hoxc6 appears able to function as a post-mitotic determinant of brachial LMC identity. Furthermore, the ability of EnR Hox derivatives to inhibit brachial LMC and CT differentiation provides the best evidence so far^{27–30} that Hox6 and Hox9 activities are required normally for MN columnar specification.

LMC neurons are generated at lumbar as well as brachial levels of the spinal cord, raising the issue of whether FGF signalling and Hox proteins have a more general role in the specification of LMC identity. *Hox10* paralogues are expressed by lumbar level LMC neurons^{31,32} and are regulated by signals from the node³¹ through the synergistic activities of FGFs and GDF11¹¹. Moreover, ectopic thoracic expression of *Hoxd10* induces RALDH2 expression in MNs (J.S.D. and T.M.J., unpublished data), supporting the idea that Hox proteins are involved in the specification of both lumbar and brachial LMC identity.

Comparative strategies of Hox function

The principles of homeodomain protein function along the rostrocaudal axis parallel those that operate along the dorsoventral axis to establish generic MN identity^{3,4}. Along both axes, the initial graded activity of a secreted signalling factor establishes broad domains of homeodomain protein expression that are refined through selective cross-repressive interactions. These two intersecting programmes of transcriptional cross-repression, however, appear to act at different stages of MN specification. Dorsoventrally, homeodomain cross-repressive interactions are evident within neural progenitor cells^{33,34}, whereas rostrocaudally Hox cross-repressive interactions occur within post-mitotic MNs. Nevertheless, the convergence of these two patterning programmes ensures that expression of RALDH2, *BMP5* and other Hox-directed features of columnar differentiation is confined to MNs.

Our findings in the spinal cord can be compared with studies on the role of *Hox* genes in the specification of MN identity in the hindbrain. Most compellingly, Hoxb1 has been shown to control the

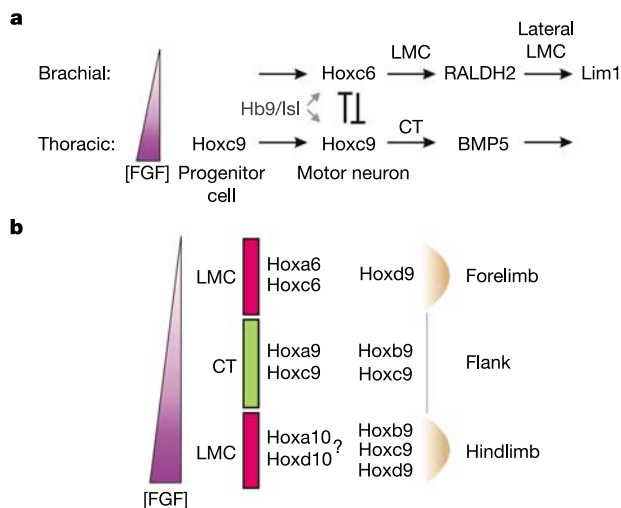


Figure 7 Hox6 and Hox9 activities in motor neuron columnar differentiation and topography. **a**, Model indicating the roles of FGF signalling and Hox6 and Hox9 expression in the specification of MN columnar identity. Hoxc9 expression in progenitors and cross-repressive interactions between Hox6 and Hox9 proteins in post-mitotic Hb9⁺, Isl⁺ MNs consolidate the distinct Hox profiles of LMC and CT neurons. Hox6 activity in brachial MNs directs RALDH2 expression and late features of LMC identity, whereas Hox9 activity in thoracic MNs directs *BMP5* expression and the dorsomedial migration of MNs. **b**, Hox expression and the register between the rostrocaudal positions of LMC and limb formation. Model suggests how exposure of neural and lateral plate mesodermal cells to a common node-derived source of FGFs could establish distinct *Hox* expression profiles in these two tissues, directing LMC and limb formation in an aligned manner. The profiles of Hox expression in lumbar LMC neurons and lateral plate mesoderm are inferred from refs 41 and 44.

differentiation of facial MNs^{13,14,35}, through both cell autonomous and non-autonomous outputs³⁶. It remains unclear, however, whether Hoxb1 controls MN specification through its activity in progenitor cells and/or post-mitotic MNs. And whether the combined activator and repressor roles suggested for individual Hox proteins in spinal MN columnar specification has a parallel in Hox function in the hindbrain remains to be determined.

The emerging logic of Hox function in spinal MN columnar diversification contrasts with certain features of *HOMC/Hox* function in rostrocaudal patterning in the *Drosophila* embryo and vertebrate hindbrain. In both systems, the actions of more posteriorly expressed Hox genes typically dominate over those of more anteriorly expressed genes—a phenomenon termed “posterior prevalence”^{37,38}. Our findings argue against a simple posterior prevalence of Hox function in post-mitotic MN specification, because ectopic caudal expression of Hoxc6 is as effective as ectopic rostral expression of Hoxc9. Exceptions to the posterior prevalence rule of Hox function have been reported in both *Drosophila* and vertebrate embryos^{37,39}, suggesting that tissue context influences the logic of Hox function.

Hox function and MN topographic organization

Our findings also provide insights into the role of Hox proteins in establishing the topographic organization of spinal MNs and their peripheral targets. One aspect of such topography is a register between the rostrocaudal positions of the LMC in the spinal cord, and of the limb field in the lateral plate mesoderm. The pathway of LMC specification revealed here has an intriguing counterpart in the proposed functions of FGFs and Hox proteins in determining limb position. Local application of FGFs to thoracic mesoderm induces the formation of an additional limb in place of body-wall mesoderm⁴⁰. Moreover, exposure of lateral plate mesoderm to FGFs induces an early switch in the rostrocaudal profile of *Hox9* paralogue expression⁴¹. Thus, exposure of neural and lateral plate mesodermal cells to a common node-derived source of FGFs could establish distinct profiles of Hox gene expression in these two tissues, profiles that in turn direct the alignment of LMC and limb formation (Fig. 7b).

Once the register between LMC neurons and limb targets has been established, the expression of Hox proteins by MNs appears to initiate more refined aspects of MN topography. The assignment of brachial LMC identity by Hox6 proteins results in the expression of RALDH2, establishing the LMC as a local source of retinoids²⁰. In turn, exposure of LMC neurons to retinoids induces the patterned expression of LIM homeodomain proteins that links MN position in the spinal cord to motor axon trajectory along the dorsoventral axis of the limb^{20,42}. Thus, the expression of Hox6 by brachial MNs initially establishes the rostrocaudal position of LMC generation, and subsequently directs dorsoventral motor topography. Conversely, the ectopic expression of Hoxc9 in brachial level MNs is associated with the redirection of their axons to sympathetic ganglion targets, an axonal trajectory characteristic of CT neurons (Supplementary Fig. S8a–d). Because the specification of CT identity by Hox9 proteins is accompanied by *BMP5* expression, it is possible that BMPs influence aspects of CT differentiation in a manner similar to the role of RALDH2 expression and retinoid signalling in LMC differentiation.

Could Hox proteins have further roles in MN diversification and topography? Within the LMC, MNs can be further subdivided into discrete pools, each destined to innervate a single muscle target in the limb⁵. The differential rostrocaudal domains of expression of the Hoxc5–Hoxc8 protein pair parallel motor pool position within the brachial LMC⁴³ (J.D. and T. J., unpublished data), and later profiles of Hox gene expression are also indicative of motor pool restriction^{10,31,32}. Although the targeted inactivation of *Hoxc8*, *Hoxa10* and *Hoxd10* in mice causes defects in motor axon projections in the limb^{44,45}, the cellular basis of these defects remains unclear. Our

findings raise the possibility that MN columnar and pool identities, and consequent patterns of motor innervation in the limb, are established through the selective activities of complementary homeodomain protein pairs embedded within individual Hox clusters. □

Methods

Expression constructs

For expression of FGF8, the murine complementary DNA was cloned into the *pCMV* cytomegalovirus (CMV)-based expression vector. Full-length Hox cDNAs were obtained by RT-PCR of embryonic day (E)10.5–E12.5 mouse or stage (st)25–st27 chick embryo total RNA using the One-Step RT-PCR kit (Invitrogen) and were confirmed by DNA sequencing. The mouse *Hoxc6* cDNA corresponded to the longer P11 transcript. cDNAs were cloned into *pCAGGS* or *pHb9* vectors by standard procedures. For generation of *Drosophila* EnR (amino acids 2–229) derivatives, cDNAs were cloned by PCR to generate in-frame fusion proteins.

In ovo electroporation

Electroporation was performed as described¹⁸. Results shown for each experiment are representative of at least eight electroporated embryos. For misexpression of Hox-c proteins in chick embryos, plasmids were titrated to achieve levels of protein expression qualitatively similar to endogenous levels. Typically this was in the range of 100–500 $\mu\text{g}\mu\text{l}^{-1}$ for the *pCAGGS* vector and 3–4 $\text{ng}\mu\text{l}^{-1}$ for the *Hb9* vector, using *CMV/GFP* or *Hb9/GFP* plasmids as carrier DNA. Similarly, the *CMV/FGF8* plasmid was titrated to a level (between 100–200 $\text{ng}\mu\text{l}^{-1}$) where somite and dorsoventral patterning and generic aspects of MN generation were unaffected.

In situ hybridization histochemistry and immunohistochemistry

In situ hybridization was performed as described¹⁷. Chick probes for analysis of *Hoxc6*, *Hoxc9*, *Hoxa9*, *Hoxb9* and *Hoxd9* expression were provided by C. Tabin. Probes against *Hoxa6* and *Hoxb6* were obtained by RT-PCR, using sequence information obtained from the chick EST data consortium. Antibodies against Hox-c proteins and LIM homeodomain proteins were used as previously described^{11,17}. Additional antibodies were obtained as follows: goat anti-Hoxa9 (Santa Cruz Biotechnology, SC-17155), mouse anti-phospho MAPK (Sigma, M8159).

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Near-infrared flares from accreting gas around the supermassive black hole at the Galactic Centre

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Recent measurements of stellar orbits^{1–3} provide compelling evidence that the compact radio source Sagittarius A* (refs 4, 5) at the Galactic Centre is a 3.6-million-solar-mass black hole. Sgr A* is remarkably faint in all wavebands other than the radio region^{6,7}, however, which challenges current theories of matter accretion and radiation surrounding black holes⁸. The black hole's rotation rate is not known, and therefore neither is the structure of space-time around it. Here we report high-resolution infrared observations of Sgr A* that reveal 'quiescent' emission and several flares. The infrared emission originates from within a few milliarcseconds of the black hole, and traces very energetic electrons or moderately hot gas within the innermost accretion region. Two flares exhibit a 17-minute quasi-periodic variability. If the periodicity arises from relativistic modulation of orbiting gas, the emission must come from just outside the event horizon, and the black hole must be rotating at about half of the maximum possible rate.

During observations of the Galactic Centre with the new Very Large Telescope adaptive optics (AO) imager NACO^{9,10} on 9 May 2003, we observed a powerful flaring by a factor of 5 in the H-band (1.65- μm) emission towards Sgr A* (Fig. 1, Table 1). The flare lasted for 30 min. Its rise and decay can be well fitted by an exponential of timescale 5 min. In a second observing run in June 2003, we observed two more flares on two consecutive days, this time in the K_s band (2.16 μm). The K-band flares rose to a factor of 3 above

the quiescent level, and each lasted for ~ 85 min. Their characteristic rise/decay times were 2 to 5 min. Both flares exhibited significant and similar temporal substructure (Fig. 2). The 16 June flare showed five major peaks spaced by 13 to 17 min, resembling a 15–40%, quasi-periodic modulation of the overall flare profile. The 15 June flare had three major peaks separated by 14 and 17 min, followed by a weaker peak 28 min later.

The power spectrum analysis for both flares (Fig. 2) exhibits a significant peak with a time period of 16.8 ± 2 min, thus confirming the reality of the structure seen directly in the light curves. The comparison star S1 clearly does not show such a quasi-periodic structure. However, the question arises whether this periodicity is truly a fundamental and significant property of all SgrA* flares¹¹, or whether it is caused by fluctuations in a 'red noise' power spectrum. More data will tell, but the fact that two events separated by about 93 periods show similar substructure is very suggestive. Because of the lack of continuous time coverage, we cannot make a statement on the substructure of the May H-band flare. Finally we found a fourth flare in re-analysing earlier archival L'-band (3.76- μm) NACO data taken on 30 August 2002. This flare rose to 70% above the quiescent emission (Fig. 2), and had a decay time of ~ 10 min. The infrared flares all originated from within a few milliarcseconds, or a few hundred Schwarzschild radii, of the black-hole position (Table 1). That position was determined from the focus of the best-fitting Kepler orbit of the star S2^{1,3}.

Before and after the strong flares and in further images taken in H, K_s and L' in August 2002 and in March, May, June and July 2003, we also detected a 'quiescent' source towards the position of SgrA* (Table 1; see also Y. Clenet, D.R., D. Gratadour, E. Gendron, F.L., A. M. Lagrange, G. Rousset, R.G. & R.S., manuscript in preparation). The source is spatially unresolved, and exhibits small-amplitude variability (20–60%) on a scale of 20–30 min. It is not clear whether this variability also has some kind of periodicity. The H-band observations of 16 June may have several peaks spaced by ~ 28 min. Similar spacings are seen in the off-flare structure on 15 and 16 June (Fig. 2). Hour-to-week-scale variability of the SgrA* L'-band emission was also discovered in May and June 2003 at the Keck telescope¹². It might thus also be appropriate to interpret the SgrA* infrared emission as exhibiting a range of variability amplitudes and timescales of which the observed flares are just the most extreme at present. Wire-grid K-band polarimetry taken on 14 June 2003 shows that the 'quiescent' source is significantly more polarized than the average of stars in its immediate vicinity. More calibration needs to be carried out before a precise value and position angle can be quoted. The quiescent source is coincident

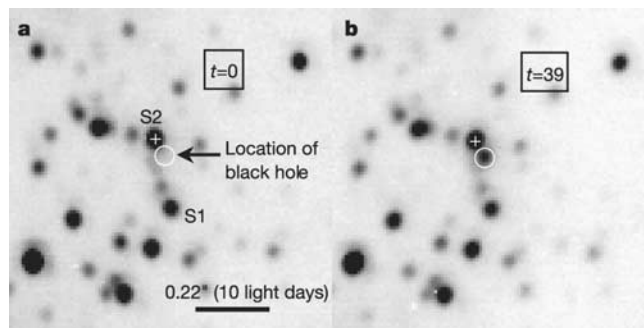


Figure 1 Detection of variable near-infrared emission from SgrA*. Raw H-band (1.65- μm) AO images (40 mas full-width at half-maximum, FWHM) of the central $\sim 1''$ of the Milky Way, obtained with the NACO AO imager^{9,10} on UT4 (Yepun) of the ESO VLT, before and during the H-band flare on 9 May 2003. The image scale is linear. The integration time for each image was 60 s, from six 10-s individual exposures. The time (in minutes from the beginning of the set at 6 h 59 min 24 s (UT) on 9 May 2003) is shown in the box in the upper right of each image. The images were sky-subtracted, flat-fielded and corrected for

bad pixels. North is up and east to the left, scales are for an assumed distance of 7.94 kpc (25,880 light years)³. The unique infrared wavefront sensor was used to close the loop of the AO system on the bright supergiant IRS7, $\sim 5.5''$ north of SgrA*. The fraction of the power in the diffraction-limited core (Strehl ratio) is about 50% (visible seeing 0.45'' FWHM). The position of the 15-yr-orbit star S2^{1–3} is marked by a cross, and the astrometric location of the black hole is marked by a circle.

Table 1 Photometry and astrometry of the near-infrared emission from SgrA*

Band	Date	ΔRA^* (mas)	ΔDec^* (mas)	$S_{\nu}^{\dagger}$ (mJy)	Duration ‡ (min)	Variability §	Period (min)
H quiescent	2002.63 to 2003.53	0 (8)	23 (8)	2.8 (0.6)		≤ 0.2 to 0.6	(28)#
K _s quiescent	2002.63 to 2003.45	-6 (8)	15 (12)	2.7 (0.6)		0.3	(25–30)#
L' quiescent	2002.66 to 2003.45	-9 (15)	-4 (20)	6.4 (1.9)		>2 between 2002 and 2003	None
				2003.21/35			
				17.5 (5) ¶			
				2002.63/.66			
H flare	2003.353	-1.4 (3)	-0.2 (3)	13 (3)	30	4.7	?
K _s flare	2003.455	-2.5 (4)	3.4 (4)	10.5 (3)	80	3.1	16.6
K _s flare	2003.457	-6.4 (4)	2.5 (4)	7.3 (3)	85	3.2	17.1
L' flare	2002.66	0 (30)	0 (30)	12.6 (4) ¶	≥ 15	0.7	None

Numbers in parentheses are ($\pm$) 1σ errors in all cases. Dates include fractions of the year.

*Relative to the best fit focus position of S2 orbit³.

† Dereddened flux density, corrected for $A(H) = 4.3$, $A(K) = 2.8$ and $A(L') = 1.8$. In the case of flares, the quiescent emission is subtracted. Errors contain statistical, systematic uncertainties and variability.

‡ Full-width zero power (FWZP).

§ Excess of variable emission relative to steady emission.

$||$ See Y. Clenet, D.R., D. Gratadour, E. Gendron, F.L., A. M. Lagrange, G. Rousset, R.G. & R.S. (manuscript in preparation) for details and calibration of L'-data.

$||$ ¶ In 2002.6 S2 and SgrA* could not be resolved in L'; for this reason the L' flux densities given are after subtraction of 8.8 mJy for S2.

Very uncertain.

with the position of the black hole to within 10–20 mas, where the accuracy is limited by its faintness and proximity to S2.

It is highly unlikely that the source is a star projected along the line of sight towards SgrA*, given that it is variable, polarized and coincident with SgrA* in four epochs between March and July. For

Poisson statistics and four detected flares over a total period of 25 h we estimate a rate between 2 and 6 flares per day, at least twice the rate of X-ray flares from Chandra monitoring in 2002 (1.2 ± 0.4 flares per day; ref. 13). This high flaring rate, along with the complex time substructure of the two K-band flares, rules out the possibility

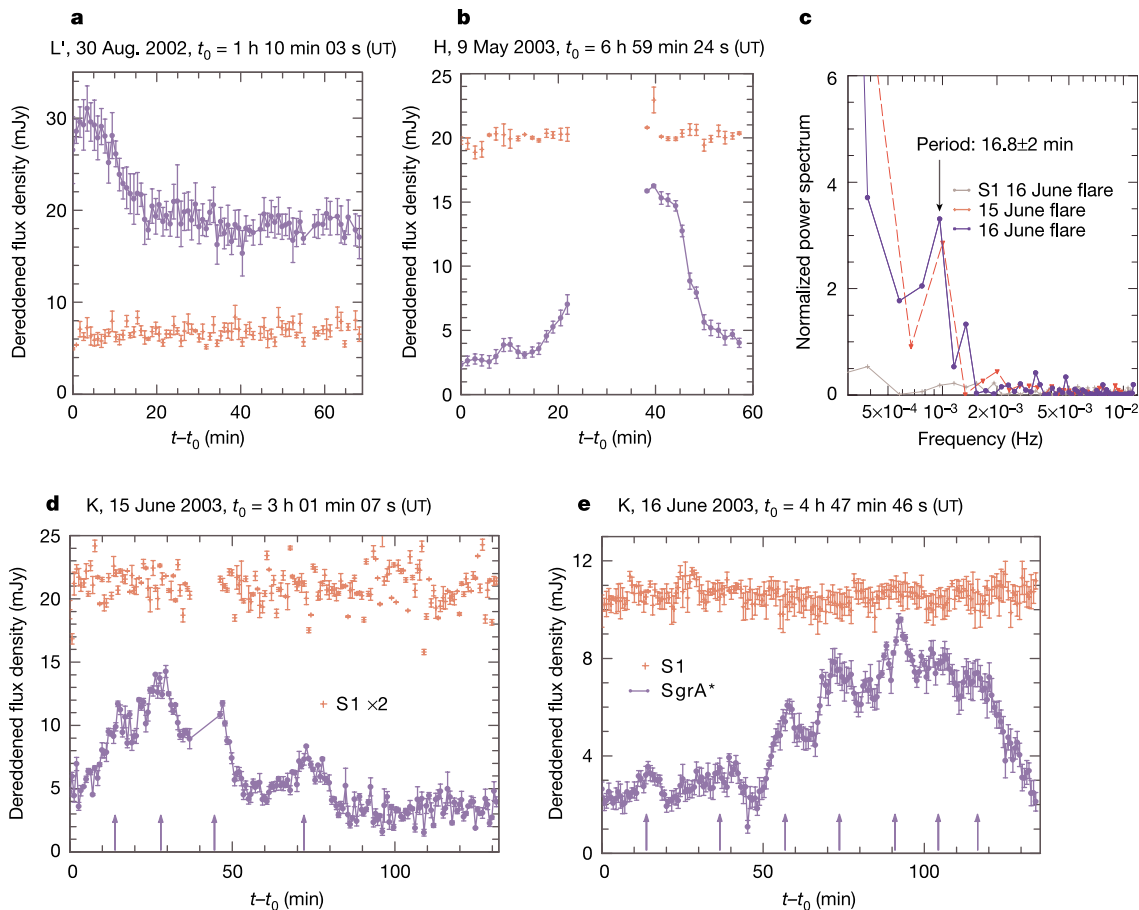


Figure 2 Light curves of the SgrA* infrared flares and quiescent emission in 2002–03. Flux densities were extracted from the Lucy deconvolved and beam restored images with two aperture sizes. Error bars ($\pm 1\sigma$) indicate the combined statistical and systematic uncertainties. SgrA* data are shown as filled blue circles (connected with a solid curve). For comparison, the light curves of the nearby star S1 are shown as light red crosses (0.2" southwest of SgrA*, left panel of Fig. 1). S1 has flux densities comparable to the SgrA* flare state. In all cases the times are relative to the UT time listed above each graph. Arrows in **d** and **e** mark the substructure peaks discussed in the text. **a**, Light curve of the 30 August L'-band flare. As S2 and SgrA* cannot be spatially

separated in this epoch, we have subtracted 8.8 mJy to account for the contribution of S2. **b**, Light curve of the 9 May H-band flare. The gap in the data between $t - t_0 = 23$ and 37 min was due to sky observations during that time. **c**, Power spectra of the flares in panels **d** and **e**, and S1, normalized by their high frequency noise. **d**, K_s-band light curve on 15 June 2003. Between $t - t_0 = 37$ and 46 min, the AO system was not operational. Because of our choice of the dichroic for the AO system, the signal-to-noise ratio of this flare is not as good as in the 16 June flare. For better presentation, the flux densities of S1 were multiplied by a factor of 2. **e**, K_s-band light curve on 16 June 2003. The time structure of this flare may have the tendency to chirp (periods decrease with time).

that the flares are due to gravitational microlensing of cusp stars by the black hole. The lensing rate is predicted to be several orders of magnitude smaller than the observed flaring rate¹⁴. It is unclear whether our high flare rate is consistent with the previous limits on the infrared activity of SgrA*. Most of the previous studies relied on speckle imaging, for which integration times of ≥ 10 min (a significant fraction of the flare duration) were required to confidently detect a source at the measured flux densities. A retroactive analysis of some of our best data in the mid-1990s gives an inconclusive answer as to whether such variability was or was not present. SgrA* may be more active at present than in the past decade.

Figure 3 summarizes the radio to X-ray spectral energy distribution (SED) of the emission from SgrA*, for both states. The quiescent infrared SED is red, with flux densities decreasing with frequency. Taking into account the variation in 'quiescent' L'-band flux from 2002 to 2003 (Table 1), the inferred 1.6–3.8 μm SED has a power-law spectral index of $-2.1 (\pm 1.4)$. As predicted by a number of models^{15–18}, the 'quiescent' infrared flux densities lie approximately on the extrapolation of the millimetre/submillimetre synchrotron emission to high energies, in accordance with a standard power-law synchrotron SED. Our detection of polarization is also consistent with the synchrotron model. Models with only a thermal population of electrons^{15,16} underpredict the infrared emission, whereas models with an additional non-thermal, power-law com-

ponent of energetic electrons ($\gamma_e = E_e/m_e c^2 \geq 10^{2.5}$, where E_e and m_e are the energy and mass of the electron; ref. 17) come closer to, but are still below, the observed emission.

The observed infrared and X-ray flare durations, rise/decay times and band luminosities are similar (Fig. 3; refs 13, 19, 20). The higher fractional flare amplitudes in X-rays are probably just a reflection of the much fainter quiescent emission. Although the millimetre/submillimetre emission of SgrA* is also variable, almost all of these variations are on timescales of several days to a few hundred days^{21,22}. One exception is a one-hour-duration, 30% amplitude event seen in March 2000 at a wavelength of 2 mm (ref. 22). Although we do not have a simultaneous SED of the infrared flares, our data suggest that they may be bluer than the quiescent emission with a flux density that is approximately constant as a function of frequency (Fig. 3). The infrared flares may be synchrotron emission as well, if turbulence, magnetic reconnection or shocks are effective in accelerating for short periods a significant fraction of the energetic electrons to $\gamma_e \geq 10^3$ (Fig. 3; ref. 17). In that case, the increased emission is due to an acceleration event, similar to a solar flare, rather than enhanced accretion^{17,18}. Models with suitably up-scaled fractions of very energetic electrons may account for the luminosities/fluxes of the infrared flares (as well as the 'quiescent' emission).

However, if the observed flare fluxes do represent the intrinsic SED of the flare, an alternative emission mechanism is required. In that case, the infrared flares may be thermal bremsstrahlung or black-body radiation from a second component of moderately hot gas (temperature in excess of a few times 10^3 K) associated with individual accretion events of very dense gas, of total energy release $\geq 10^{39.5}$ erg (for an assumed radiation efficiency of $\sim 10\%$, this energy requires an accreted mass of a few times 10^{19} g, comparable to that of a comet or a small asteroid), while the observed radio/submillimetre emission in that case would come from a jet or be optically very thick. A key future test of the two alternatives will be measurements of the simultaneous infrared flare SED and polarization. The flares' location close to the central black hole, as well as the temporal substructure, poses a serious challenge to models in which the flares originate from rapid shock cooling of a large-scale jet, or are due to passages of stars through a central accretion disk²³.

The few-minute rise and decay times, as well as the quasi-periodicity, strongly suggest that the infrared flares originate in the innermost accretion zone, on a scale less than ten Schwarzschild radii (the light travel time across the Schwarzschild radius of a 3.6-million-solar-mass black hole (1.06×10^{12} cm) is 35 s). If the substructure is a fundamental property of the flow, the most likely interpretation of the periodicity is the relativistic modulation of the emission of gas orbiting in a prograde disk just outside the last stable orbit (LSO)²⁴. If the 17-min period can be identified with this fundamental orbital frequency, the inevitable conclusion is that the Galactic Centre black hole must have significant spin. The LSO frequency of a 3.6-million-solar-mass, non-rotating (Schwarzschild) black hole is 27 min. Because the prograde LSO is closer in for a rotating (Kerr) black hole, the observed period can be matched if the spin parameter is $J/(GM_{\text{BH}}/c) = 0.52 (\pm 0.1, \pm 0.08, \pm 0.08)$, where J is the angular momentum of the black hole; this is half the maximum value for a Kerr black hole^{25,26}. (The error estimates here reflect the uncertainties in the period, black-hole mass (M_{BH}) and distance to the Galactic Centre³, respectively; G is the gravitational constant.) For that spin parameter, the last stable orbit is at a radius of 2.2×10^{12} cm. Recent numerical simulations of Kerr accretion disks indicate that the in-spiralling gas radiates most efficiently just outside the innermost stable orbit²⁷. Our estimate of the spin parameter is thus a lower bound.

Other possible periodicities, such as acoustic waves in a thin disk²⁸, Lense-Thirring or orbital node precession are too slow for explaining the observed frequencies for any spin parameter²⁵. (The 28-min timescale of the quiescent emission corresponds to a radius

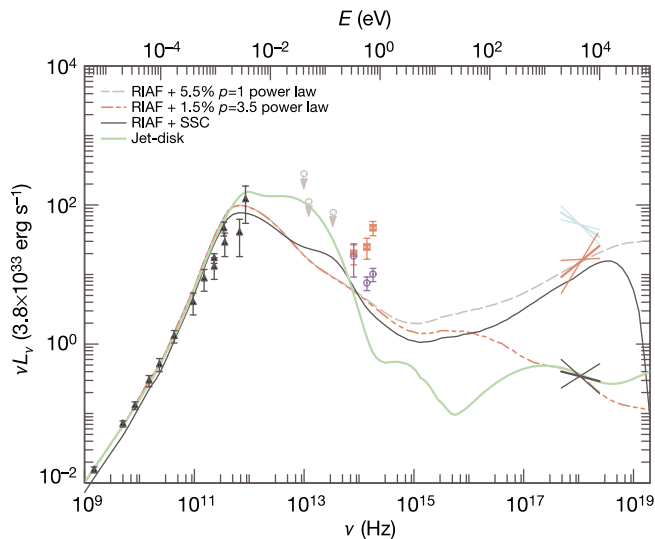


Figure 3 Spectral energy distribution emission from SgrA*. Plotted are the extinction and absorption corrected, band luminosities νL_ν (energy emitted per logarithmic energy interval) as a function of frequency, or energy. The observed flux density is $S_\nu = L_\nu/4\pi D^2$, where $D = 7.94$ kpc is the Galactic Centre distance. All error bars are $\pm 1\sigma$. Black triangles denote the quiescent radio spectrum of SgrA*^{18,21}. Open grey circles denote various infrared upper limits from the literature¹⁸. The three X-ray data ranges are (from bottom to top) the quiescent state as determined with Chandra⁶ (black), the autumn 2000 Chandra flare¹⁹ (red) and the autumn 2002 XMM flare²⁰ (light blue). Open red squares with crosses mark the peak emission (minus quiescent emission) observed in the four flares (Table 1; note that the measurements were taken at different times). Open blue circles denote the dereddened H, K_s and L' luminosities of the quiescent state, derived from the local background subtracted flux density of the point source at the position of SgrA*, thus eliminating the contribution from any extended diffuse light from the stellar cusp around the black hole. Values plotted are from Table 1 (average of 2002–03 in the case of L' band). The thick green solid curve is the jet-starved disk model¹⁵. The red long dash-short dash curve is a radiatively inefficient accretion flow (RIAF) model of the quiescent emission, where in addition to the thermal electron population of ref. 15, 1.5% of the electrons are in a non-thermal power-law energy spectrum of exponent $p = -3.5$ (ref. 17). The black thin solid curve is a RIAF model of the flares with 5.5% of the electrons in a power law of $p = -1$ (ref. 17). The long-dash blue curve is a RIAF flare model of the flares with a synchrotron-self Compton component¹⁷.

of 3.2×10^{12} cm for a prograde orbit of $J/(GM_{\text{BH}}/c) = 0.52$; the last stable retrograde orbit for that spin parameter has a period of 38 min at a radius of 4×10^{12} cm). Lense-Thirring precession and viscous (magnetic) torques will gradually force the accreting gas into the black hole's equatorial plane²⁹. Recent numerical simulations indicate that a (prograde) disk analysis is appropriate to first order even for the hot accretion flow at the Galactic Centre²⁷. □

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Controlled collisions for multi-particle entanglement of optically trapped atoms

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Entanglement lies at the heart of quantum mechanics, and in recent years has been identified as an essential resource for quantum information processing and computation^{1–4}. The experimentally challenging production of highly entangled multi-particle states is therefore important for investigating both fundamental physics and practical applications. Here we report the creation of highly entangled states of neutral atoms trapped in the periodic potential of an optical lattice. Controlled collisions between individual neighbouring atoms are used to realize an array of quantum gates, with massively parallel operation. We observe a coherent entangling–disentangling evolution in the many-body system, depending on the phase shift acquired during the collision between neighbouring atoms. Such dynamics are indicative of highly entangled many-body states; moreover, these are formed in a single operational step, independent of the size of the system^{5,6}.

Bose–Einstein condensates have been loaded into the periodic dipole force potential of a standing-wave laser field—a so-called optical lattice. In these systems, it has been possible to probe fundamental many-body quantum mechanics in an unprecedented way, with experiments ranging from Josephson junction tunnel arrays^{7,8} to the observation of a Mott insulating state of quantum gases^{9,10}. Important applications of atoms in a Mott insulating state in quantum information processing were envisaged early on. The Mott state itself, with one atom per lattice site, could act as a huge quantum memory, in which information would be stored in atoms at different lattice sites. Going beyond these ideas, it has been suggested that controlled interactions between atoms on neighbouring lattice sites could be used to realize a massively parallel array of neutral-atom quantum gates^{5,11–14}, with which a large multi-particle system could be highly entangled⁶ in a single operational step. Furthermore, the repeated application of the quantum gate array could form the basis for a universal quantum simulator along the original ideas of Feynman for a quantum computer as a simulator of quantum dynamics^{15–17}.

The basic requirement for such control over the quantum state of a many-body system, including its entanglement, is the precise microscopic control of the interactions between atoms on different lattice sites. To illustrate this, let us consider the case of two neighbouring atoms, initially in state $|\Psi\rangle = |0\rangle_j |0\rangle_{j+1}$ placed on the j th and $(j+1)$ th lattice site of the periodic potential in the spin-state $|0\rangle$. First, both atoms are brought into a superposition of two internal states $|0\rangle$ and $|1\rangle$, using a $\pi/2$ pulse such that $|\Psi\rangle = (|0\rangle_j + |1\rangle_j)(|0\rangle_{j+1} + |1\rangle_{j+1})/2$. Then, a spin-dependent transport¹⁸ splits the spatial wave packet of each atom such that the wave packet of the atom in state $|0\rangle$ moves to the left, whereas the wave packet of the atom in state $|1\rangle$ moves to the right. The two wave packets are separated by a distance $\Delta x = \lambda/2$, such that now $|\Psi\rangle = (|0\rangle_j |0\rangle_{j+1} + |0\rangle_j |1\rangle_{j+2} + |1\rangle_{j+1} |0\rangle_{j+1} + |1\rangle_{j+1} |1\rangle_{j+2})/2$, where in the notation atoms in state $|0\rangle$ have retained their original lattice site index and λ is the wavelength of the laser forming the optical periodic potential. The collisional interaction between the atoms^{5,12,19} over a time t_{hold} will lead to a distinct phase shift $\varphi = U_{01} t_{\text{hold}}/\hbar$, when

both atoms occupy the same lattice site $j + 1$ resulting in: $|\Psi\rangle = (|0\rangle_j|0\rangle_{j+1} + |0\rangle_j|1\rangle_{j+2} + e^{-i\varphi}|1\rangle_{j+1}|0\rangle_{j+1} + |1\rangle_{j+1}|1\rangle_{j+2})/2$. Here U_{01} is the onsite-interaction matrix element that characterizes the interaction energy when an atom in state $|0\rangle$ and an atom in state $|1\rangle$ are placed at the same lattice site and $\hbar$ is Planck's constant divided by 2π . Alternatively, a dipole-dipole interaction has been proposed¹¹ for generating a state-dependent phase shift φ . The final many-body state after bringing the atoms back to their original site and applying a last $\pi/2$ pulse can be expressed as $|\Psi\rangle = \frac{1+e^{-i\varphi}}{2}|1\rangle_j|1\rangle_{j+1} + \frac{1-e^{-i\varphi}}{2}|\text{BELL}\rangle$. Here $|\text{BELL}\rangle$ denotes the Bell-like state corresponding to $(|0\rangle_j|0\rangle_{j+1} - |1\rangle_{j+1}) + |1\rangle_j(|0\rangle_{j+1} + |1\rangle_{j+1})/2$.

This scheme can be generalized when more than two particles are placed next to each other, starting from a Mott insulating state of matter^{9,10}. In such a Mott insulating state, atoms are localized to lattice sites, with a fixed number of atoms per site. For three particles for example, one can show that if $\varphi = (2n + 1)\pi$ (with n being an integer), so-called maximally entangled Greenberger–Horne–Zeilinger (GHZ) states²⁰ are realized. For a string of $N > 3$ atoms, where each atom interacts with its left- and right-hand neighbour (see Fig. 1), the entire string of atoms can be entangled to form so-called cluster states in a single operational step^{5,6}. The controlled interactions described above can be viewed as being equivalent to an ensemble of quantum gates acting in parallel^{3,5}.

The experimental set-up used to load Bose–Einstein condensates into the three-dimensional optical lattice potential (see Methods section) is similar to our previous work^{10,19}. Briefly, we start with a quasi-pure Bose–Einstein condensate of 10^5 ^{87}Rb atoms in the $|F = 1, m_F = -1\rangle$ state in a harmonic magnetic trapping potential with isotropic trapping frequencies of $\omega = 2\pi \times 14$ Hz. Here F and m_F denote the total angular momentum and the magnetic quantum number of the atom's hyperfine state. The three-dimensional periodic potential of an optical lattice is then ramped up over a period of 80 ms to a potential depth of $25E_r$, such that the Bose–Einstein condensate is converted into a Mott insulating state. Here

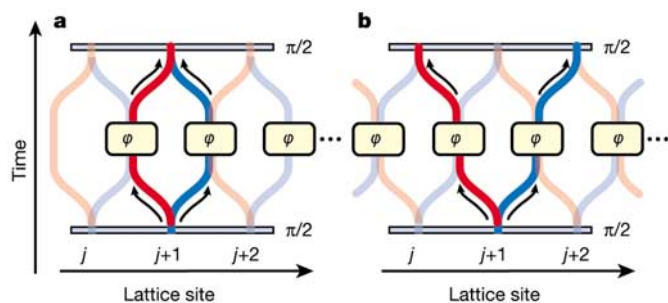


Figure 1 Schematic multiple quantum gate sequences based on controlled interactions. **a**, A chain of neutral atoms on different lattice sites is first placed in a coherent superposition of two spin-states $|0\rangle$ (red) and $|1\rangle$ (blue) with a $\pi/2$ microwave pulse. Then a spin-dependent transport is used to split the spatial wave packet of an atom, and move these two components along two opposite directions depending on their spin-state. The wave packets are separated by a lattice period such that each atom is brought into contact with its neighbouring atom. Owing to the collisional interaction between the atoms, a phase shift φ is acquired during a time t_{hold} that the atoms are held on a common lattice site depending on the spin-state of the atoms. After such a controlled collisional interaction, the wave packets of the individual atoms are returned to their original site and a final microwave $\pi/2$ pulse is applied to all atoms. This multiple quantum gate sequence can be equivalently described as a controllable quantum Ising interaction^{6,12}. **b**, In a slight modification of such a sequence, the atoms are not returned to their original lattice site $j + 1$ but rather delocalized further over the j th and $(j + 2)$ th lattice site after the controlled collisional interaction. The small arrows indicate the different paths that a single atom will follow during the multiple quantum gate sequence. Both sequences can be viewed as multi-particle interferometers, where the many-body output state of the interferometer can in general not be expressed as a product state of single-particle wavefunctions.

E_r denotes the recoil energy $E_r = \hbar^2 k^2 / 2m$, with $k = 2\pi/\lambda$ being the wavevector of the laser light and m the mass of a single atom. For our experimental parameters of atom number and harmonic confinement, such a Mott insulator should consist mainly of a central core with $n = 1$ atoms per lattice site^{9,21,22}. The magnetic trapping potential is then rapidly switched off, but an actively stabilized magnetic offset field of 1 G along the transport direction is maintained to preserve the spin polarization of the atoms. With the optical standing wave along this direction, we are able to realize a spin-dependent transport of the atoms. After turning off the magnetic trapping field, we wait another 40 ms for the electronics to stabilize the magnetic offset field. Thereafter, 3.5 ms before the quantum gate sequence is initiated, we adiabatically increase the lattice depth along this axis to $34 E_r$ such that atoms remain in the vibrational ground state, are tightly confined and can be moved as fast as possible without excitations to higher vibrational states.

In the experiment, the two hyperfine states $|F = 1, m_F = -1\rangle \equiv |0\rangle$ and $|F = 2, m_F = -2\rangle \equiv |1\rangle$ form the logical basis of a single-atom qubit at each lattice site. These two states can be coupled

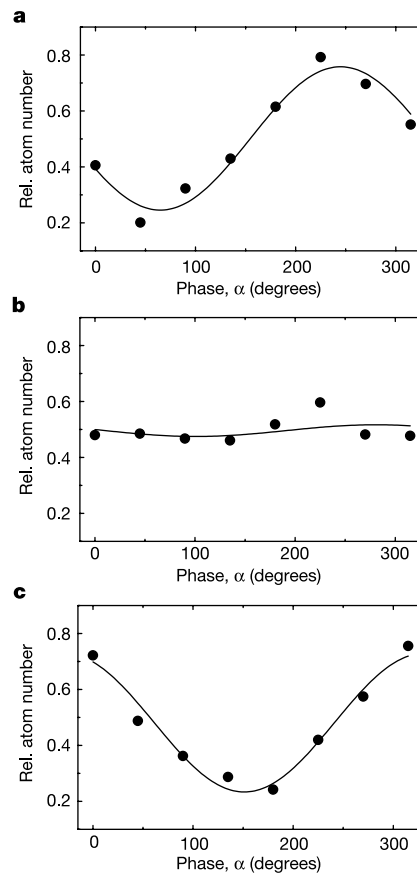


Figure 2 Experimentally measured Ramsey fringes for different hold times t_{hold} during which atoms undergo a controlled collisional interaction with their neighbouring atoms. The experimental sequence used is similar to the one in Fig. 1a, where atoms are returned to their original lattice site after the controlled interaction. The hold times t_{hold} are **a**, 30 μs , **b**, 210 μs and **c**, 450 μs . The relative number of atoms $N_{\text{rel}} = N_1/N_{\text{tot}}$ in the $|1\rangle$ state versus the phase α of the final microwave $\pi/2$ pulse is measured. A state-selective absorption imaging of the atom cloud is used to obtain N_1 after a time-of-flight period of 12 ms, and 110 μs thereafter the total atom number is measured to yield N_{tot} . The solid line indicates a fit of a sinusoidal function with variable amplitude and an offset to the data from which the visibility of the Ramsey fringe is extracted. The change in the phase of the Ramsey fringes for different hold times is mainly caused by the different exposure times of the two spin-states of an atom to differential light shifts of the optical lattice that are not perfectly cancelled in the spin-echo sequence.

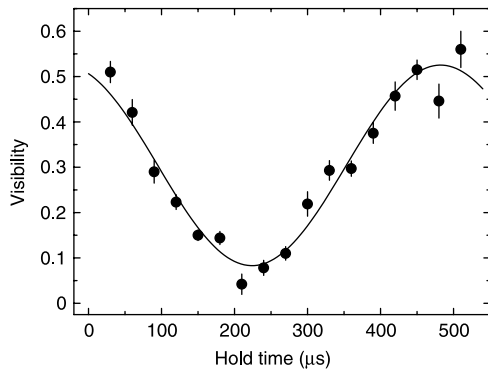


Figure 3 Visibility of Ramsey fringes versus hold times on neighbouring lattice sites for the experimental sequence similar to the one displayed in Fig. 1a. The solid line is a sinusoidal fit to the data including an offset and a finite amplitude. Such a sinusoidal behaviour of the visibility versus the collisional phase shift (determined by the hold time t_{hold}) is expected for a Mott insulating state with an occupancy of $n = 1$ atom per lattice site²³. The maximum observed visibility is limited to 55% by inhomogeneities and time-dependent fluctuations of the lattice potentials throughout the cloud of atoms that are not perfectly compensated by the applied spin-echo sequence (see text).

coherently using resonant microwave radiation around 6.8 GHz. A $\pi/2$ pulse allows us to place the atom in a coherent superposition of the two states within a time of 6 μs . After creating such a coherent superposition, we use a spin-dependent transfer to split and move the spatial wavefunction of the atom over half a lattice spacing in two opposite directions depending on its internal state (see Fig. 1). Such a movement process is carried out within a time of 40 μs in order to avoid any vibrational excitations¹⁸ (the probability for excitations into higher-lying vibrational states was measured to be less than 3%). Atoms on neighbouring sites then interact for a variable amount of time t_{hold} . After half of the hold time, a microwave π pulse is applied. This spin-echo type π pulse is mainly used to cancel unwanted single-particle phase shifts, due, for example, to inhomogeneities in the trapping potentials. It does not, however, affect the non-trivial and crucial collisional phase shift due to the interactions between the atoms. After such a controlled collision, the atoms are moved back to their original site. Then a final $\pi/2$ microwave pulse with variable phase α is applied, and the atom number in state $|1\rangle$ relative to the total atom number is recorded.

The Ramsey fringes obtained in this way are shown in Fig. 2 for some different hold times t_{hold} , and for a wider range of hold times their visibility is plotted in Fig. 3. For short hold times, where no significant collisional phase shift is acquired, a Ramsey fringe with a

visibility of approximately 50% is recorded. For longer hold times we notice a strong reduction in the visibility of the Ramsey fringe, with an almost vanishing visibility of approximately 5% for a hold time of 210 μs (Fig. 2b). This hold time corresponds to an acquired collisional phase shift of $\varphi = \pi$ for which we expect a minimum visibility if the system is becoming entangled.

For such an entangled state the probability for finding atoms in state $|1\rangle$ becomes independent of the phase α corresponding to a vanishing Ramsey fringe. This can be seen, for example, for the two-particle case: when the phase α of the last pulse is kept variable, the maximally entangled state for a collisional phase $\varphi = (2n + 1)\pi$ can be expressed as: $|\Psi(\varphi = \pi)\rangle = \frac{1}{\sqrt{2}}(|0\rangle|-\rangle + |1\rangle|+\rangle)$, where $|-\rangle, \alpha\rangle \equiv \frac{1}{\sqrt{2}}(c_-^-|0\rangle - c_-^-|1\rangle)$ and $|+\rangle, \alpha\rangle \equiv \frac{1}{\sqrt{2}}(c_+^+|0\rangle + c_+^+|1\rangle)$ with $c_c^\pm \equiv e^{\pm i\alpha}\cos\alpha$ and $c_s^\pm \equiv -(\pm i\sin\alpha - 1)$. Here the probability for finding an atom in either spin-state, for example, $P(|1\rangle)$, is independent of α and equal to 1/2: $P(|1\rangle) = \frac{1}{8}\{|c_+^+|^2 + |c_-^-|^2 + 2|c_c^+|^2\} = \frac{1}{2}$. This indicates that no single-particle operation can place all atoms in either spin-state when a maximally entangled state has been created. The disappearance of the Ramsey fringe has been shown to occur not only for a two-particle system, but is a general feature for an arbitrary N -particle array of atoms that have been highly entangled with the above experimental sequence^{3,23}. A vanishing Ramsey fringe can therefore in principle not distinguish between two-particle or multi-particle entanglement.

For longer hold times, the visibility of the Ramsey fringe increases again reaching a maximum of 55% for a hold time of 450 μs . Here the system becomes disentangled again, as the collisional phase shift is close to $\varphi = 2\pi$ and the Ramsey fringe is restored with maximum visibility.

The coherent ‘entanglement oscillations’ of the many-body system⁶ are recorded for longer hold times by using the multi-particle interferometer sequence of Fig. 1b, where the atoms are not brought back to their original site but are rather kept delocalized¹⁸. This allows us to observe the Ramsey fringe of the previous sequence as a spatial interference pattern in a single run of the experiment in analogy to a double-slit interference experiment, when a state-selective time-of-flight detection is used. Images of such an interference pattern can be seen in Fig. 4 for different hold times t_{hold} . The coherent evolution again indicates the entangling–disentangling dynamics that the system undergoes for different collisional phase shifts φ (see Fig. 5).

Although the observed coherent dynamics in the vanishing and re-emergence of the Ramsey fringe does not provide a rigorous proof of a highly entangled multi-particle state, it is very indicative of such a state. So far, we cannot employ single-atom measurement techniques to detect correlations between individual atoms in the cluster that would provide a quantitative measurement for the size

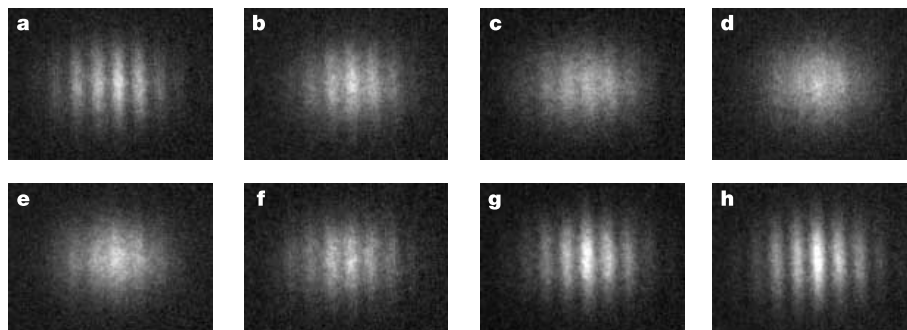


Figure 4 Spatial interference patterns recorded after applying the multiple quantum gate sequence of Fig. 1b for different collisional interaction times t_{hold} . The different hold times (μs) of 30 (a), 90 (b), 150 (c), 210 (d), 270 (e), 330 (f), 390 (g) and 450 (h) lead to different collisional phase shifts φ , ranging from $\varphi \approx 0$ (a) to just over $\varphi \approx 2\pi$ (h). The

vanishing and reappearance of the interference pattern is caused by the coherent entangling–disentangling dynamics in the many-body system due to the controlled collisions between neighbouring atoms. The state-selective absorption images were obtained after a time-of-flight period of 11 ms.

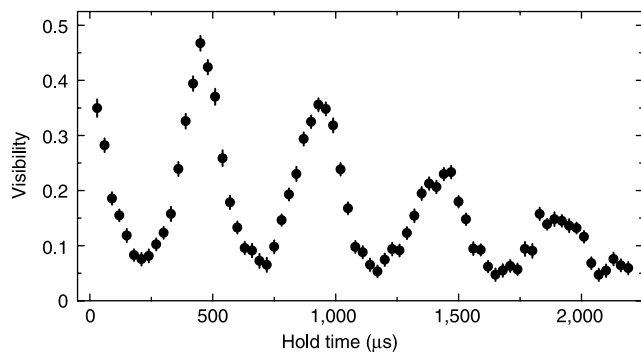


Figure 5 Visibility of the spatial interference patterns versus different collisional interaction times t_{hold} . We have been able to observe up to four entangling–disentangling cycles in the experiment. The reduced visibility for longer hold times is mainly caused by a dephasing over the trapped cloud of atoms due to inhomogeneities in the external potentials.

of the entangled many-body state. It is clear, however, that the minimum visibility observed in the Ramsey fringes is dependent on the quality of our initial Mott insulating state and the fidelity of the quantum gate operations. In an ideal experimental situation with perfect fidelity for the multi-particle quantum gates and a defect-free Mott insulating state, this visibility should vanish for a phase shift of $\varphi = (2n + 1)\pi$. For a finite fidelity of the quantum gates, caused, for example, by a 5% fractional error in the pulse areas of the microwave pulses, the minimum visibility would already increase to $\sim 2\%$. If defects are present in the initial quantum state of the Mott insulator—for example, vacant lattice sites—then the entangled cluster state will not extend beyond this vacancy and the visibility of the Ramsey fringe will become non-zero owing to isolated atoms in the lattice. We have noticed, for example, that the quality of the Mott insulating state deteriorates owing to its prolonged uncompensated exposure to the potential gradient of gravity after the magnetic trapping potential is turned off. In addition to an imperfect creation of the Mott state, such vacancies could be caused by the superfluid shell of atoms surrounding the Mott insulating core^{9,21,22} or spontaneous emission due to the laser light, which leads to excitations of approximately 5% of the atoms for our total experimental sequence times.

In our one-dimensional lattice shift the system is very susceptible to vacant lattice sites, as a defect will immediately limit the size of the cluster. However, the scheme can be extended to two or three dimensions by using two additional lattice shift operations along the remaining orthogonal lattice axes. As long as the filling factor of lattice sites exceeds the percolation threshold (31% for a three-dimensional simple cubic lattice system²⁴) a large entangled cluster should be formed, making massive entanglement of 100,000 atoms possible in only three operational steps. For some of the applications of such a highly entangled state it will, however, be crucial to locate the position of the defects in the lattice.

In the future, it will be interesting to explore schemes for quantum computing that are based only on single-particle operations and measurements on such a cluster state². Here the large amount of entanglement in a cluster state can be viewed as a resource for quantum computations. But now, even without the possibility of manipulating single atoms in the periodic potential, a quantum computer based on the controlled collisions demonstrated here could be able to simulate a wide class of complex hamiltonians of condensed-matter physics that are translationally invariant^{12,17}. □

Methods

Optical lattices

A three-dimensional array of microscopic potential wells is created by overlapping three orthogonal optical standing waves at the position of the Bose–Einstein condensate. In our

case the atoms are trapped in the intensity maxima of the standing-wave light field owing to the resulting dipole force^{25,26}. The laser beams for two of the periodic potentials are operated at a wavelength of $\lambda = 820$ nm with beam waists of approximately $210 \mu\text{m}$ at the position of the Bose–Einstein condensate. This gaussian laser beam profile leads to an additional isotropic harmonic confinement of the atoms with trapping frequencies of 40 Hz for lattice potential depths of $25E_r$. In this configuration, we populate almost 100,000 lattice sites with an average atom number per lattice site of up to 1 in the centre of the lattice. The lattice structure is of simple cubic type, with a lattice spacing of $\lambda/2$ and oscillation frequencies in each lattice potential well of approximately 30 kHz for a potential depth of $25 E_r$.

State-dependent lattice potentials

Along a third orthogonal direction a standing-wave potential at a wavelength of $\lambda_x = 785$ nm is used, formed by two counter-propagating laser beams with linear polarization vectors^{5,11,18}. The angle θ between these polarization vectors can be dynamically adjusted through an electro-optical modulator and additional polarization optics. Such a lin-angle-lin polarization configuration can be decomposed into a σ^+ and a σ^- polarized standing-wave laser field, giving rise to potentials $V_+(x, \theta) = V_0 \cos^2(k_x x + \theta/2)$ and $V_-(x, \theta) = V_0 \cos^2(k_x x - \theta/2)$. Here V_0 is the potential depth of the lattice. By changing the polarization angle θ one can control the separation $\Delta x = (\theta/\pi)(\lambda_x/2)$ between the two potentials. When increasing θ , both potentials shift in opposite directions and overlap again for $\theta = n\pi$. For our experimental conditions, the dipole potential experienced by atoms in the $|1\rangle$ state is given by $V_-(x, \theta)$ and for atoms in the $|0\rangle$ state, it is dominated by the $V_+(x, \theta)$ potential¹⁸. For these laser beams, a waist of $150 \mu\text{m}$ has been used, resulting in a maximum potential depth of $34E_r$ and corresponding maximum vibrational trapping frequencies of 39 kHz.

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Demonstration of conditional gate operation using superconducting charge qubits

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Following the demonstration of coherent control of the quantum state of a superconducting charge qubit¹, a variety of qubits based on Josephson junctions have been implemented^{2–5}. Although such solid-state devices are not currently as advanced as microscopic qubits based on nuclear magnetic resonance⁶ and ion trap⁷ technologies, the potential scalability of the former systems—together with progress in their coherence times and read-out schemes—makes them strong candidates for the building block of a quantum computer⁸. Recently, coherent oscillations⁹ and microwave spectroscopy¹⁰ of capacitively coupled superconducting qubits have been reported; the next challenging step towards quantum computation is the realization of logic gates^{11,12}. Here we demonstrate conditional gate operation using a pair of coupled superconducting charge qubits. Using a pulse technique, we prepare different input states and show that their amplitude

can be transformed by controlled-NOT (C-NOT) gate operation, although the phase evolution during the gate operation remains to be clarified.

A Cooper-pair box provides an artificial two-level system, where two charge states, say $|0\rangle$ and $|1\rangle$, differing by $2e$ of one Cooper pair (e is the electronic charge) are coherently superposed by Josephson coupling¹³. When two Cooper-pair boxes are connected by a capacitor, the quantum states of the boxes interfere with each other. This results in quantum beating, as has been demonstrated recently⁹. Using this coherent four-level system formed by the charge states $|00\rangle$, $|01\rangle$, $|10\rangle$ and $|11\rangle$, we show how to implement a logic gate and demonstrate that it works as a quantum gate.

A scanning electron micrograph of the sample is shown in Fig. 1a. Two qubits are electrostatically coupled by an on-chip capacitor⁹. The right qubit has SQUID (superconducting quantum interference device) geometry, and we use this qubit as the control qubit and the left one as the target qubit. Unlike the previous coupled-qubit sample⁹, there are two independent pulse gates so that we can address each qubit individually. This is essential to the logic operation, as explained below.

In the two-qubit charge basis $|00\rangle$, $|10\rangle$, $|01\rangle$ and $|11\rangle$, the hamiltonian of the system is given as

$$H = \sum_{n_1, n_2=0,1} E_{n_1 n_2} |n_1, n_2\rangle \langle n_1, n_2| - \frac{E_{J1}}{2} \sum_{n_2=0,1} (|0\rangle \langle 1| + |1\rangle \langle 0|) \otimes |n_2\rangle \langle n_2| - \frac{E_{J2}}{2} \sum_{n_1=0,1} |n_1\rangle \langle n_1| \otimes (|0\rangle \langle 1| + |1\rangle \langle 0|), \quad (1)$$

where E_{J1} (E_{J2}) is the Josephson coupling energy of the first (second) box to the reservoir and $E_{n_1 n_2} = E_{c1}(n_{g1} - n_1)^2 +$

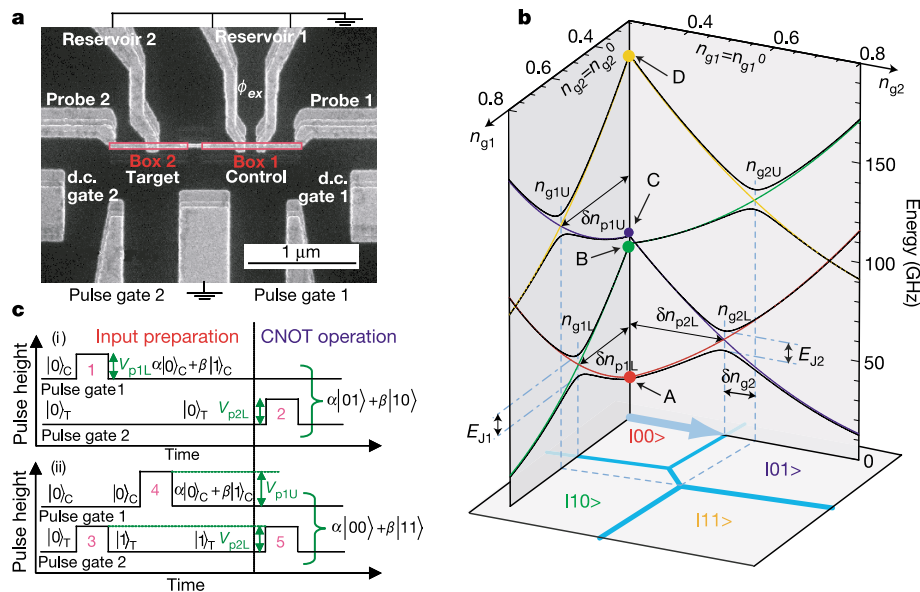


Figure 1 Pulse operation of the coupled-qubit device. **a**, Scanning electron micrograph of the sample. The qubits were fabricated by electron-beam lithography and three-angle evaporation of Al on a SiN_x insulating layer above a gold ground plane on the oxidized Si substrate. The two strips enclosed by red lines are the Cooper-pair boxes, which are coupled by an on-chip capacitor⁹. ϕ_{ex} represents magnetic flux penetrating the SQUID loop. An electrode between the two pulse gates is connected to the ground to reduce the cross capacitance. Although there is a finite cross capacitance between one gate and the other box (about 15% of the main coupling), it does not play any essential role in the present experiment and so we can neglect it in this Letter. The sample was cooled to 40 mK in a dilution refrigerator. The characteristic energies of this sample estimated from the d.c. current–voltage measurements are $E_{c1} = 580 \mu\text{eV}$, $E_{c2} = 671 \mu\text{eV}$ and $E_m = 95 \mu\text{eV}$. From the pulse measurements, E_{J1} is found to be $45 \mu\text{eV}$ at a maximum

and E_{J2} to be $41 \mu\text{eV}$. The superconducting energy gap is $209 \mu\text{eV}$. Probe junction tunnel resistance is equal to $48 \text{ M}\Omega$ (left) and $33 \text{ M}\Omega$ (right). **b**, Energy band diagram along two lines of $n_{g1} = n_{g1}^0$ and $n_{g2} = n_{g2}^0$, where n_{g1}^0 and n_{g2}^0 are constants. Here $(n_{g1}^0, n_{g2}^0) = (0.24, 0.26)$, corresponding to the actual experimental condition. In the energy band diagram, black lines show the eigenenergies. The four coloured lines are the charging energies of the states shown in the cells of the charging diagram of the base plane with the corresponding colour. **c**, Pulse sequences used in the experiment. In both sequences (i) and (ii), the upper and lower patterns show the pulse sequences applied to pulse gates 1 and 2, respectively. The expected quantum states after each pulse are also shown. The symbols $|0\rangle$ or $|1\rangle$ with subscripts C and T mean the state of the control and target qubits, respectively.

$E_{c2}(n_{g2} - n_2)^2 + E_m(n_{g1} - n_1)(n_{g2} - n_2)$ is the total electrostatic energy of the system ($n_1, n_2 = 0, 1$ is the number of excess Cooper pairs in the first and second boxes, and $n_{g1,2}$ are the gate-induced charges on the corresponding qubit divided by $2e$). $E_{c1(2)} = 4e^2 C_{\Sigma 1(2)} / 2(C_{\Sigma 1} C_{\Sigma 2} - C_m^2)$ are the effective Cooper-pair charging energies ($C_{\Sigma 1(2)}$ are the sum of all capacitances connected to the corresponding island including the coupling capacitance C_m between two boxes). Finally, $E_m = 4e^2 C_m / (C_{\Sigma 1} C_{\Sigma 2} - C_m^2)$ is the coupling energy. In our notation of $|n_1, n_2\rangle$ for the charge basis, n_1 and n_2 represent the states of the control and target qubits, respectively.

Figure 1b represents the idea for the gate operation. Using equation (1), we calculate the eigenenergies of the two-qubit system and plot them in the planes $n_{g1} = n_{g1}^0$ and $n_{g2} = n_{g2}^0$, where n_{g1}^0 and n_{g2}^0 are constants. In these planes, if (n_{g1}^0, n_{g2}^0) is sufficiently far away from the co-resonant point⁹ (0.5, 0.5), four energy bands can be regarded as two pairs of nearly independent single-qubit energy bands. In the plane of $n_{g1} = n_{g1}^0$, for example, our system is divided into a pair of independent two-level systems $|00\rangle, |01\rangle$ and $|10\rangle, |11\rangle$. Importantly, the charging energies of each of the two-level systems degenerate at different n_{g2} , namely, at n_{g2L} for the states $|00\rangle$ and $|01\rangle$ and at n_{g2U} for the states $|10\rangle$ and $|11\rangle$, as shown in Fig. 1b. This difference (δn_{g2}) originates from the electrostatic coupling between the qubits, and is given as $E_m/2E_{c2}$. Similarly, we define n_{g1L} and n_{g1U} as shown in the plane of $n_{g2} = n_{g2}^0$.

Now we consider the pulse operation. Applying pulses to pulse gate 1 (2) shifts the system non-adiabatically in the plane of $n_{g2} = n_{g2}^0$ ($n_{g1} = n_{g1}^0$). For convenience, we define the distances from (n_{g1}^0, n_{g2}^0) to the degeneracy points as follows: $\delta n_{p1L} = n_{g1L} - n_{g1}^0$, $\delta n_{p1U} = n_{g1U} - n_{g1}^0$ and $\delta n_{p2L} = n_{g2L} - n_{g2}^0$. Suppose we start from the $|00\rangle$ state (point A) and apply an ideal rectangular pulse with an amplitude $V_{p2L} = 2e \delta n_{p2L} / C_{p2}$ to pulse gate 2, where C_{p2} is the capacitance between pulse gate 2 and box 2. This pulse is represented by the arrow in the ground-state charging diagram¹⁴ of the base plane. In this case, the system is brought to the degeneracy point n_{g2L} and evolves during a pulse duration Δt with a frequency $\Omega = E_{J2}/\hbar$ between the $|00\rangle$ and the $|01\rangle$ states: $\cos(\Omega\Delta t/2)|00\rangle + \sin(\Omega\Delta t/2)|01\rangle$. By adjusting Δt so that $\Omega\Delta t = \pi$ (π -pulse), we can stop the evolution when the system is in the $|01\rangle$ state. The system is finally in the state at point C after the termination of the pulse.

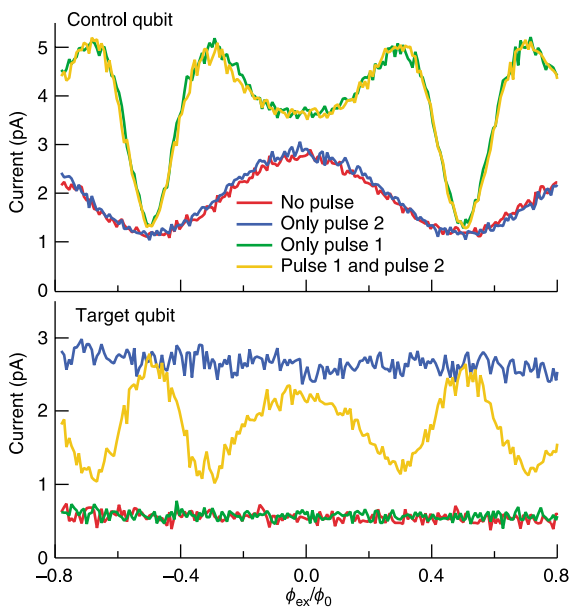


Figure 2 Magnetic-flux dependence of the current of the control (top) and target (bottom) qubits under the application of pulses shown in Fig. 1c (i). The lengths of the pulses are $\Delta t_1 = 85$ ps, $\Delta t_2 = 255$ ps and $\Delta t_{12} = 85$ ps, where we define the pulse length of pulse m in Fig. 1c as Δt_m and the interval between pulses l and m as Δt_{lm} .

On the other hand, if we start from the $|10\rangle$ state (point B) and apply the same pulse, the system does not reach the degeneracy point for states $|10\rangle$ and $|11\rangle$ (n_{g2U}). In this case, the amplitude of the oscillation between the $|10\rangle$ and the $|11\rangle$ states is suppressed by $E_{J2}^2/(E_m^2 + E_{J2}^2)$. If E_m is sufficiently large, the state $|10\rangle$ remains almost unchanged (except for the phase factor), coming back to point B after the termination of the pulse. Similarly, we can realize the transition from the $|01\rangle$ state to the $|00\rangle$ state by the same pulse, and suppress the transition out of the $|11\rangle$ state. Therefore, conditional gate operation can be carried out based on this operation pulse: the target bit is flipped only when the control bit is $|0\rangle$.

To experimentally demonstrate the above gate operation, we prepare different input states from the ground state $|00\rangle$ by applying pulses and measure the output of the gate operation. Figure 1c shows two pulse sequences that are used in the present experiment. For convenience, each of the pulses in the sequences is labelled by an index m ($m = 1, \dots, 4, 5$), which we will refer to as 'pulse m '. In sequence (i) of Fig. 1c, a superposition of the states $|00\rangle$ and $|10\rangle$ is created by applying pulse 1 with the amplitude $V_{p1L} = 2e \delta n_{p1L} / C_{p1}$, where C_{p1} is the capacitance between pulse gate 1 and box 1. In sequence (ii) of Fig. 1c, a superposition of the states $|01\rangle$ and $|11\rangle$ is created by two sequential pulses. First, pulse 3, the same pulse as that for the gate operation, brings the system to the $|01\rangle$ state at point C. Then, pulse 4 with amplitude $V_{p1U} = 2e \delta n_{p1U} / C_{p1}$ is applied.

In both sequences, an operation pulse (pulse 2 or 5) creating an

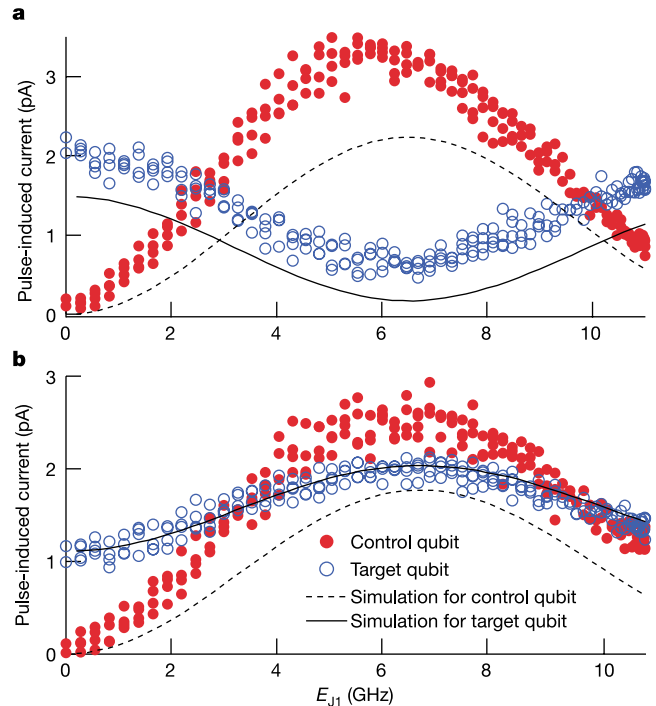


Figure 3 Pulse-induced current as a function of the Josephson energy of the control qubit. Pulse sequences used are **a**, that shown in Fig. 1c (i), and **b**, that shown in Fig. 1c (ii). The lengths of the pulses in Fig. 1c (ii) are $\Delta t_3 = 264$ ps, $\Delta t_4 = 88$ ps, $\Delta t_5 = 264$ ps, $\Delta t_{34} = 88$ ps and $\Delta t_{45} = 88$ ps. The black curves represent the simulation obtained by calculating the time evolution of the density matrix. In the calculation, we assumed a trapezoidal pulse shape with both rise and fall times equal to 40 ps, which is close to the real pulse shape. To take into account the effect of dephasing, all the off-diagonal terms of the density matrix are set to zero before applying the operation pulse. This is a reasonable approximation because the dephasing time at an off-degeneracy point is reported to be a few hundred picoseconds¹⁶, which is comparable to the time needed for the input preparation for the present experiment. We did not take into account the energy relaxation, which is known to be much slower.

entangled state ($\alpha|01\rangle + \beta|10\rangle$ or $\alpha|00\rangle + \beta|11\rangle$) is applied after the preparation pulses. To change the coefficients α and β , we change the Josephson energy of the control qubit E_{J1} by a magnetic field, while keeping the pulse lengths constant. Because the control qubit has SQUID geometry, E_{J1} is periodically modulated as $E_{J1} = E_{J1\max}|\cos(\pi \phi_{\text{ex}}/\phi_0)|$, where $E_{J1\max}$ is the maximum value of E_{J1} and ϕ_0 is the flux quantum. By repeatedly applying the sequential pulses (with a repetition time $T_r = 128$ ns), we measure the pulse-induced currents through probes 1 and 2, which are biased at ~ 650 μV to enable a Josephson-quasiparticle (JQP) cycle¹⁵. These currents are proportional to the probability of the respective qubit having one extra Cooper pair^{1,9}.

Figure 2 shows the output currents of the control qubit (I_C) and the target qubit (I_T) as a function of ϕ_{ex}/ϕ_0 under the application of pulses shown in Fig. 1c (i). When no pulse is applied, both qubits show a finite current owing to the finite width of the JQP peak (red curves in Fig. 2). Because this current depends on the Josephson energy, I_C is periodically modulated by ϕ_{ex} . First, we determine the length of the operation pulse (pulse 2) by adjusting it to the peak in the single-qubit oscillation of I_T . When we apply pulse 2 of this length (blue curves in Fig. 2), I_T is enhanced and does not depend on ϕ_{ex} , as was expected. Also, this pulse has no effect on I_C . Next, we apply the preparation pulse (pulse 1) only. This pulse, in turn, induces current in I_C while not affecting I_T (green curves in Fig. 2).

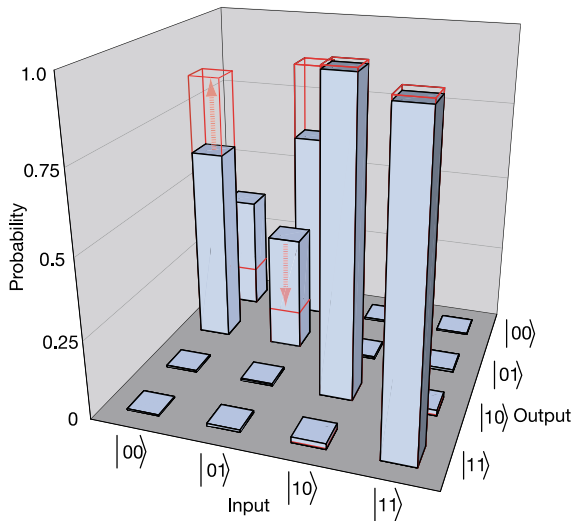


Figure 4 Truth table of the present C-NOT operation estimated by the numerical calculation (solid blue bars). Detailed values of the probabilities are

$$\begin{pmatrix} 0.37 & 0.62 & 0.004 & 0.003 \\ 0.62 & 0.37 & 0.004 & 0.007 \\ 0.004 & 0.004 & 0.97 & 0.018 \\ 0.003 & 0.007 & 0.018 & 0.97 \end{pmatrix}$$

Ideally, they should be

$$\begin{pmatrix} 0 & 1 & 0 & 0 \\ 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix}$$

We can partly see the correspondence of this figure to the experimental data in Fig. 3. Because the prepared input state in sequence (i) of Fig. 1c is almost pure $|00\rangle$ state when E_{J1} equals zero, the I_T at $E_{J1} = 0$ in Fig. 3a normalized by the possible maximum current $2e/T_r$ (2.5 pA) should be close to 0.62 (the second element of the first column of the above truth table). The experimental data gives a slightly larger value of ~ 0.8 . This is attributed to the leak current discussed in the text. The red lines and arrows indicate the expected improvement after decreasing the rise/fall time of the pulses from 40 to 30 ps.

Furthermore, the magnitude of the induced current depends on ϕ_{ex} , indicating that input states with different coefficients α and β are prepared. Finally, we apply both pulse 1 and pulse 2 with an interval of 85 ps (orange curves in Fig. 2). In this case, I_C shows the same dependence as that when only pulse 1 is applied. However, I_T also shows clear dependence on ϕ_{ex} and is anti-correlated with I_C as the target qubit feels the state of the control qubit. In Fig. 3a, we re-plot this data as a function of E_{J1} . We present only pulse-induced currents by subtracting the d.c. background currents from each curve. Both I_T and I_C show cosine-like dependence but their phases are opposite. That is, I_T is maximal when I_C is minimal, and vice versa. This is consistent with the expectation that the state $\alpha|01\rangle + \beta|10\rangle$ is created by the pulse sequence used.

Next we measure the ϕ_{ex} dependence of I_C and I_T for pulse sequence (ii) of Fig. 1c (not shown) and plot it as E_{J1} dependence in Fig. 3b. In this case, as in Fig. 3a, I_T and I_C show cosine-like dependence. However, most importantly, their correlation is now opposite to that in Fig. 3a. This is consistent with the expectation that the state $\alpha|00\rangle + \beta|11\rangle$ is created.

The above data show that we have succeeded with the conditional gate operation. However, to understand our results more quantitatively, we compare the data with simulation data obtained by numerically calculating the time evolution of the density matrix. The results of the simulation are shown as black curves in Fig. 3. We stress that no fitting parameters are used in the calculation.

First, we consider the target qubit. Apart from the offset in Fig. 3a, the simulated curves agree well with the experiment, suggesting that the oscillation amplitude of the measured I_T is reasonable. Second, in contrast, we have some discrepancy in I_C . We attribute this discrepancy to the unknown current channel in our present read-out scheme. As long as the JQP process is considered, the pulse-induced current should not be able to exceed $2e/T_r = 2.5$ pA, but in reality it does. This means that the pulse-induced current has an extra component that does not originate from the JQP process. We do not yet know the origin of this current. It may be other processes involving higher-order Cooper-pair tunnelling. The magnitude of this current probably depends on the Josephson energy (but does not depend strongly on the pulse length), and produces the E_{J1} -dependent deviation between the simulated and measured curves. In the target qubit, the similar current channel simply gives a constant offset in Fig. 3 as E_{J2} is fixed and does not affect the overall E_{J1} -dependence. Although quantitative analysis for I_C is difficult at present, the simulation suggests that the oscillation amplitude of the measured I_T is reasonable, whereas that of I_C is enhanced by this extrinsic factor originating from the imperfection of our read-out scheme.

Last, we estimate the accuracy of our gate operation and propose possible ways for improvement. Our present read-out scheme, which does not allow us to measure the probability of the four states individually⁹, makes it difficult to obtain the complete truth table of our gate operation solely from the experimental data. Instead, here we do it on the basis of the simulation that turned out a reasonable description of our two-qubit system, as shown in Fig. 3. We calculate the time evolution of four perfect input states, $|00\rangle$, $|01\rangle$, $|10\rangle$ and $|11\rangle$, under the application of the operation pulse, namely pulse 2 or 5 in Fig. 1c, and plot the output probabilities as solid blue bars in Fig. 4. For the input states of $|10\rangle$ and $|11\rangle$, our gate operation is almost ideal. Note that the accuracy is better than that expected for the case of the ideal pulse shape, that is, $1 - E_{J2}^2/(E_m^2 + E_{J2}^2) \approx 0.84$. This is due to the finite rise/fall time (40 ps) of the operation pulse, which suppresses the unwanted oscillation. On the other hand, for the input states of $|00\rangle$ and $|01\rangle$, the output states have an unwanted component of $|00\rangle$ or $|01\rangle$ with a rather high probability. This is also due to the finite rise/fall time, which in this case suppresses the desired oscillation. To improve this, increasing E_m as well as making the pulse shape ideal would be the best solution. However, even with the present value of

E_m , the simulation suggests that this matrix becomes much closer to the ideal one (keeping almost ideal outputs for $|10\rangle$ and $|11\rangle$ input states) if we slightly decrease the rise/fall time, say by 25% (red lines in Fig. 4), or decrease E_{J2} by a similar amount.

We controlled our two-qubit solid-state circuit by applying a sequence of pulses, and demonstrated the conditional gate operation. Although in the present experiment we paid attention only to the amplitude of the quantum state, phase evolution during the gate operation should also be examined for the realization of the quantum C-NOT gate (probably with additional phase factors), which is a constituent of the universal gate. □

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High-*Q* photonic nanocavity in a two-dimensional photonic crystal

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Photonic cavities that strongly confine light are finding applications in many areas of physics and engineering, including coherent electron–photon interactions¹, ultra-small filters^{2,3}, low-threshold lasers⁴, photonic chips⁵, nonlinear optics⁶ and quantum information processing⁷. Critical for these applications

is the realization of a cavity with both high quality factor, *Q*, and small modal volume, *V*. The ratio *Q/V* determines the strength of the various cavity interactions, and an ultra-small cavity enables large-scale integration and single-mode operation for a broad range of wavelengths. However, a high-*Q* cavity of optical wavelength size is difficult to fabricate, as radiation loss increases in inverse proportion to cavity size. With the exception of a few recent theoretical studies^{8–10}, definitive theories and experiments for creating high-*Q* nanocavities have not been extensively investigated. Here we use a silicon-based two-dimensional photonic-crystal slab to fabricate a nanocavity with *Q* = 45,000 and *V* = $7.0 \times 10^{-14} \text{ cm}^3$; the value of *Q/V* is 10–100 times larger than in previous studies^{4,11–14}. Underlying this development is the realization that light should be confined gently in order to be confined strongly. Integration with other photonic elements is straightforward, and a large free spectral range of 100 nm has been demonstrated.

The *Q* of a cavity is determined by the energy loss per cycle versus the energy stored. With no absorption by the cavity material, *Q* is determined by the reflection loss at the interface between the interior and exterior of the cavity. Total internal reflection (TIR) and/or Bragg reflection are generally used for light confinement. For a cavity with a size much larger than the wavelength of light, a very high *Q* has already been achieved^{14,15}. In that case, the behaviour of light confined in a large cavity obeys ray optics theory, and each ray of light reflected at the interface can be designed to fulfil TIR or Bragg reflection conditions. For much smaller cavities, deviation from ray optics becomes serious, and *Q* is greatly reduced. Light confined in a very small cavity consists of numerous plane wave components with wavevectors (**k**) of various magnitudes (*k*) and directions owing to the localization of light. As it is difficult to design all such plane wave components to obey TIR or Bragg reflection conditions, photonic nanocavities with very high *Q* factors have yet to be realized.

One of the best approaches to resolving the problem is the extension of the Bragg reflection effect in multiple directions. Structures having a two- or three-dimensional (2D or 3D) periodic change of refractive index on the scale of the light wavelength are required for such extension. These are known as photonic crystals, from an analogy to solid crystals^{5,16}. For a 3D photonic crystal, Bragg reflection conditions can be fulfilled for all the propagation directions of light in a certain frequency range, known as the photonic bandgap. A small disorder or defect introduced into the 3D photonic crystal would become an ultimate photonic nanocavity, with ultra-large *Q/V*. However, 3D photonic crystals with sufficiently strong optical confinement have yet to be created⁵.

A cavity surrounded by a 2D photonic crystal is considered a feasible solution. A 2D photonic-crystal slab, as shown in Fig. 1a, with a thickness of the order of the light wavelength is very promising, owing to strong optical confinement for both in-plane and vertical directions^{2,3}. The photonic-bandgap effect is used for light confinement in the in-plane direction, and TIR, at the interface between the slab and the air clad, in the vertical direction. Apparently, fulfilment of the TIR condition in the vertical direction is crucial in designing high-*Q/V* cavities.

To investigate vertical confinement in 2D photonic-crystal slabs, we first consider a simplified model (Fig. 2a), where the cavity consists of a dielectric material with thickness *T* and length *L*. Both sides of the cavity are closed by perfect mirrors, confining light in the *x* direction. The structure is assumed to be uniform in the *y* direction for simplicity. Light is confined by TIR in the *z* direction by the air clad, as discussed above. Figure 2b shows an example of the electric field profile inside a cavity with a very short length, 2.5λ , where λ is the resonant wavelength of light in the cavity.

The strength of the vertical (*z*-direction) confinement by TIR can be investigated by decomposing the electric field inside a cavity into a set of plane wave components with various **k**-vectors by spatial

Fourier transformation (FT), which is a similar approach to that reported in ref. 10. When the tangential component of the $\mathbf{k}$ -vector ($|k_{\parallel}|$ or $|k_x|$) of each plane wave lies within the range 0 to $2\pi/\lambda_0$ (where λ_0 is the wavelength of light in air), the wave can escape from the cavity to the air clad, because the conservation law for $|k_{\parallel}|$ (or Snell's law in the broad sense) is satisfied at the interface between the cavity and air clad, which leads to weak vertical confinement. Note that $|k_{\parallel}|$ in the air clad can take a value from 0 to $2\pi/\lambda_0$ depending on the propagation direction in the x - z plane, while $|k_{\parallel}|$ in the cavity takes a variety of values owing to the localization of light, as explained before. When $|k_{\parallel}|$ in the cavity is larger than $2\pi/\lambda_0$, the $|k_{\parallel}|$ conservation law does not hold at the interface, and light becomes strongly confined inside the cavity, which leads to strong vertical confinement. Figure 2c shows the spatial FT spectra of the electric field of Fig. 2b, where the leaky region ($|k_{\parallel}|$ is smaller than $2\pi/\lambda_0$) is indicated. Large components exist inside the leaky region, which indicates that large radiation loss occurs in the cavity.

We now consider the loss mechanism in more detail. The electric field profile inside the cavity can be expressed as a product of a fundamental sinusoidal wave with wavelength λ , and an envelope function $F(x)$ that is determined by the cavity structure. The fundamental wave gives a delta functional FT spectrum with peaks at $k = \pm 2\pi/\lambda$, while the envelope function modifies the spectrum. In the case of Fig. 2b, the envelope function is $F(x) = 1$ (for $x = -L/2$ to $L/2$) and $F(x) = 0$ (for all other x), and the corresponding FT spectrum is a sinc function with a width of about $2\pi/L$ (Fig. 2c). Although the peak of the spectrum originating from the fundamental wave is outside the leaky region, an abrupt change in the envelope function at the edges ($x = -L/2, L/2$) generates large components inside the leaky region, leading to large radiation loss. The smaller the cavity, the more serious the edge effect, drastically decreasing the Q factor.

This gives an important hint for suppressing radiation loss: the spatial variation of the envelope function at the cavity edges should not be abrupt but gentle, so that the FT spectrum does not have components inside the leaky region. On the basis of this idea, we

have used a gaussian function for $F(x)$, as shown schematically in Fig. 2d; the calculated FT spectrum is shown in Fig. 2e. The situation has drastically changed: there are very small components inside the leaky region, when compared with Fig. 2c. This suggests that the Q factor can be increased significantly by tailoring the envelope function while keeping the mode volume small.

A physical design of a high- Q photonic nanocavity has thus been carried out using a 2D photonic-crystal slab (Fig. 1b and c). The base structure is composed of Si with a triangular lattice of air 'rods' with lattice constant a ($= 0.42 \mu\text{m}$). The thickness of the slab and the radii of the air rods are $0.6a$ ($0.25 \mu\text{m}$) and $0.29a$ ($0.12 \mu\text{m}$), respectively. We made the initial structure of the cavity with three missing air rods in a line¹⁷ (Fig. 1b). With this structure, light can be confined by Bragg reflection for the in-plane directions. For the z direction, light is confined by the air clad.

The electric field profile (E_y) of the fundamental mode of the cavity at the centre plane of the slab is shown in Fig. 3a. We used 3D finite-difference time-domain methods for the calculation. Unlike the model discussed in Fig. 2, x - and y -directional (2D) FT spectra are necessary for the investigation of the vertical confinement, as light is confined two-dimensionally in the cavity. For the same reason, the TIR condition (or $k_{\parallel}$ conservation law) should be expanded two-dimensionally. Considering in-plane 2D propagation, the TIR condition is broken for plane waves having $k_{\parallel}$ inside a circle of diameter $2\pi/\lambda_0$.

Figure 3b shows the FT spectra corresponding to Fig. 3a, where the leaky region is inside the grey circle. The FT spectrum contains large components inside the leaky region. As discussed, we consider

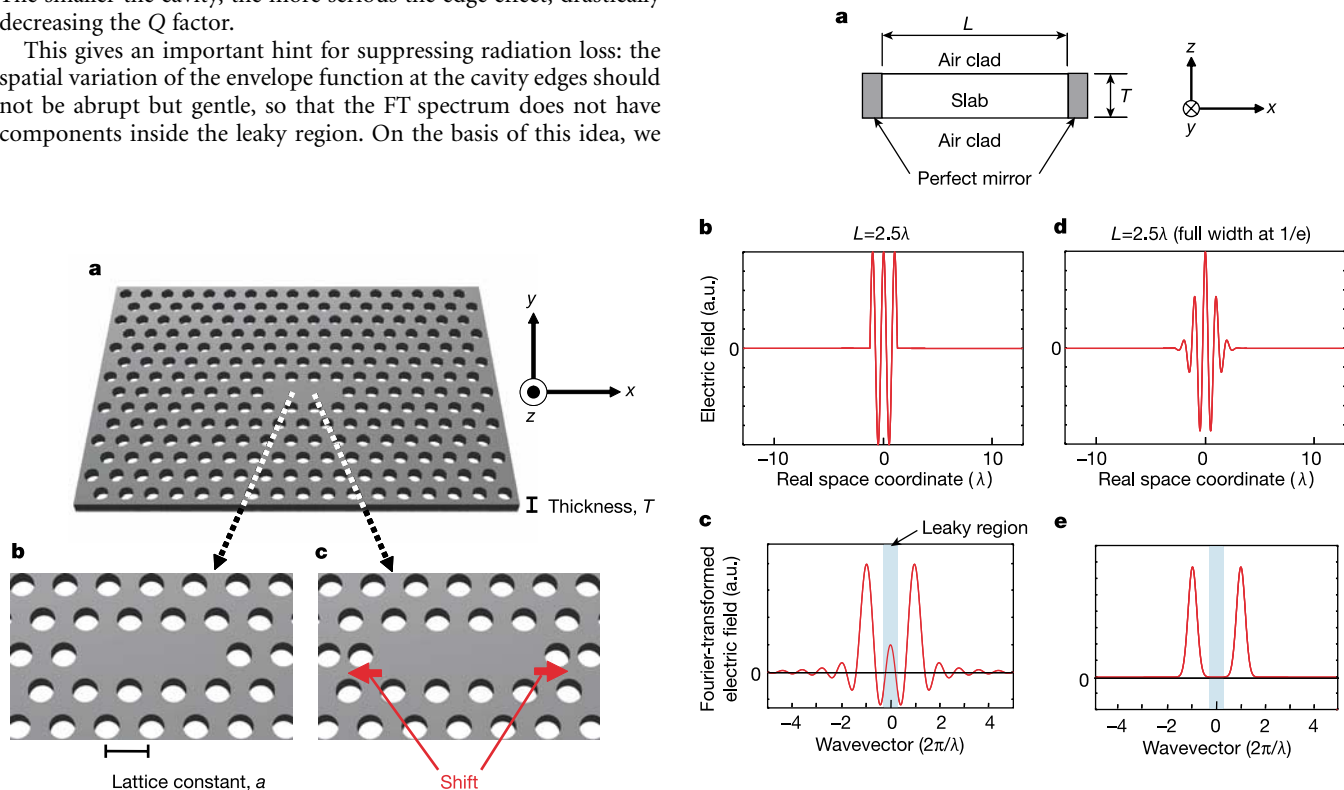


Figure 2 Analysis and reduction of cavity loss. **a**, Simplified model of a cavity consisting of a dielectric material with thickness T and length L . For confinement of light, both sides of the cavity are closed by perfect mirrors for the x direction, and by the air clad based on TIR for the z direction. **b**, **c**, The electric field profile inside a cavity with a very short (2.5λ) length, and the spatial FT spectra. The leaky region is indicated as a blue area. **d**, **e**, The electric field profile with a gentle envelope function (gaussian curve) and its spatial FT spectrum. a.u., arbitrary units.

Figure 1 Photonic nanocavities using a 2D photonic-crystal slab. **a**, Schematic of the base cavity structure having a triangular lattice of air rods with lattice constant a ($= 0.42 \mu\text{m}$). The thickness T of the slab and the radius R of the air rods are $0.6a$ ($0.25 \mu\text{m}$) and $0.29a$ ($0.12 \mu\text{m}$), respectively. **b**, Starting cavity structure with three missing air rods in a line. **c**, Designed cavity structure created by displacing the air rods at both edges to obtain an ultrahigh Q/V value.

that this is due to the abrupt change at the cavity edges. Here we try to make confinement gentler. The strategy to obtain gentler confinement is to change the condition for Bragg reflection at the cavity edge. Such reflection is determined by a summation of partial reflections at a series of rods near the cavity edge. When we move several rods near the cavity edge, the Bragg reflection condition should be modified. Because the phases of partial reflections at the moved rods are changed, the resultant phase-mismatch weakens the magnitude of Bragg reflection. To compensate for the reduction of the reflection, light is considered to penetrate more inside the mirror and be reflected perfectly. It means that the electric field profile at the cavity edge becomes gentler. With the appropriate movement of rods, the profile is considered to be close to the ideal confinement expressed by the gaussian function as discussed above. Using this strategy, the air rods at both edges of the cavity are shifted (Fig. 1c). Figure 3c and d show the electric field profile and 2D FT spectrum, respectively, where the shift of the air rods is $0.15a$ from their original position. As in Fig. 3d, the FT spectrum contains much smaller components inside the leaky region compared with Fig. 3b. The mode volume itself is confirmed as almost unchanged. Therefore, a significant increase of Q/V is expected to be achieved by this method.

Encouraged by the above analysis, we fabricated samples with various displacements. The resonant spectra were measured using a tunable c.w. laser as a light source. The cavities were excited through a line defect waveguide constructed by filling a row of air holes near the cavity (Fig. 4b), and the intensity of the light emitted from the cavities to free space was observed. Details of construction and experimental methods are given elsewhere¹⁷. The intrinsic Q factor of the cavity was determined from its radiation spectra by removing the effect of coupling between the cavity and the waveguide. The effective modal volume was calculated from the electric field profile of the cavity⁴; from this it was found that V is small and constant at $(6-7) \times 10^{-14} \text{ cm}^3$.

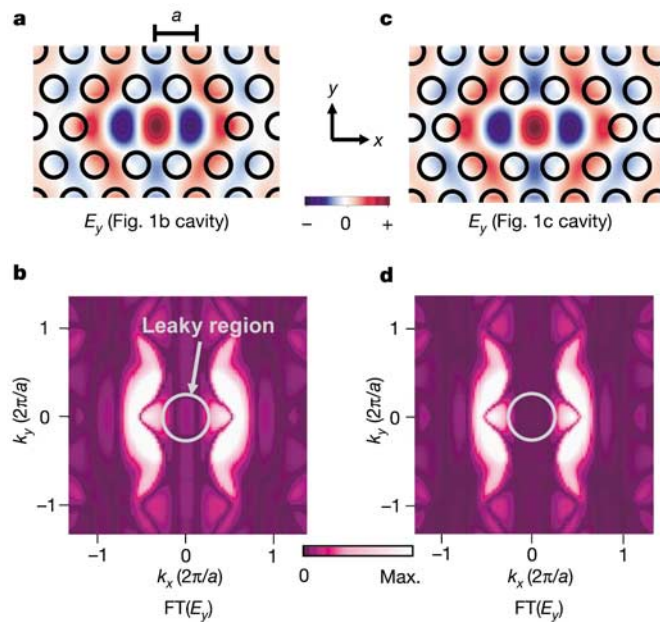


Figure 3 Physical design of high- Q/V cavity. **a**, The electric field profile (E_y) of the fundamental mode of the cavity shown in Fig. 1b as the starting structure. **b**, The FT spectra of **a**. The region inside the grey circle corresponds to the leaky region. **c**, **d**, The electric field profile and 2D FT spectrum, respectively, for the designed cavity shown in Fig. 1c. The displacement of the air rods at the edges is set at $0.15a$ from the starting structure shown in Fig. 1b.

Figures 4a and b show resonant spectra of cavities with various air-rod shifts and the corresponding scanning electron microscope (SEM) pictures, respectively. The width of the resonant peak changes drastically with shift of air rods. The spectral width becomes a minimum (0.045 nm) for the sample with shift $\sim 0.15a$, from which a Q factor of 45,000 is derived considering the coupling effect with the waveguide. In Fig. 4c, the Q/V values are plotted as a function of shift of air rods. Q/V increases by a factor of 10 upon increasing the air-rod shift up to $\sim 0.15a$. A Q/V as large as $6.4 \times 10^{17} \text{ cm}^{-3}$ or $120,000/\lambda^3$ has thus been obtained. This is one to two orders of magnitude higher than values for previously reported cavities such as toroid microcavities, microdisks and photonic-crystal cavities^{4,11-14}. Further increases of Q/V should be possible by fine-tuning the arrangement of air rods at the cavity edges to obtain the perfect gaussian curve for light confinement. The inset of Fig. 4a shows the spectrum measured for a wide wavelength range, indicating that no other resonant peak exists in the range 1,500 to 1,600 nm. The result shows that single-mode operation is possible for a broad range of wavelengths, which is very useful for various applications.

We have described the important design rule that light should be confined gently to obtain high Q factors while maintaining a very small modal volume V . An extremely large Q/V value has been achieved by introducing displacement of air rods at both edges of a cavity in a 2D photonic-crystal slab. We believe that this concept could be applied to the design of various types of photonic nanocavity; such high- Q nanocavities could be applied across various fields of science and engineering, including nano-lasers, nonlinear optics, nano-biomaterials, atom physics, and quantum computing. The present result is also important for the field of

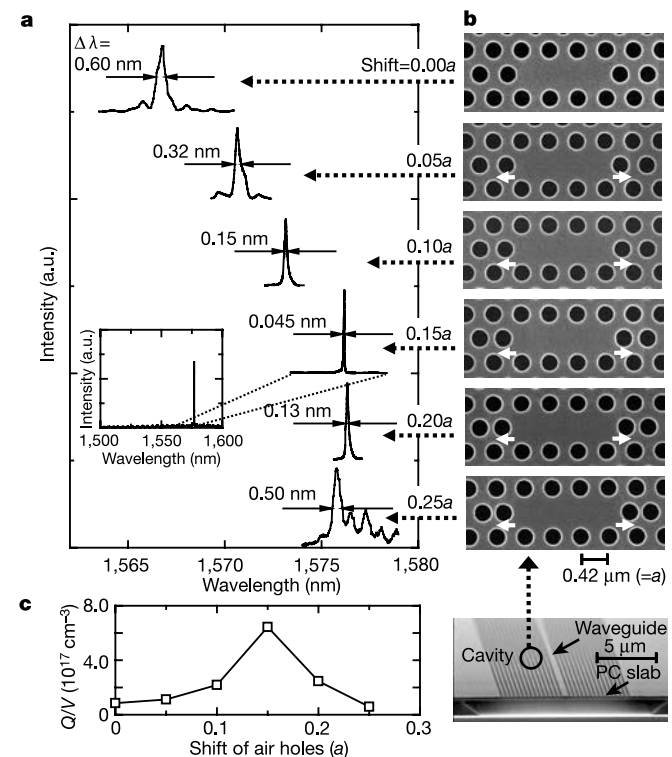


Figure 4 Experimental results. **a**, **b**, Resonant spectra of cavities with various shifts of air rods and their SEM pictures, respectively. PC, photonic crystal. The inset in **a** shows the resonant spectrum of the cavity (with $0.15a$ displacement) measured over a wide wavelength range. **c**, The estimated Q/V values as a function of shift of air rods. A maximum value of $Q/V = 6.4 \times 10^{17} \text{ cm}^{-3}$, or $120,000/\lambda^3$, has been realized.

photonic-crystal-based integrated circuits, as strong 3D confinement of photons in an ultra-small cavity has been realized, and leakage in the vertical direction sufficiently suppressed. □

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High interannual variability of sea ice thickness in the Arctic region

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Possible future changes in Arctic sea ice cover and thickness, and consequent changes in the ice-albedo feedback, represent one of the largest uncertainties in the prediction of future temperature rise^{1,2}. Knowledge of the natural variability of sea ice thickness is therefore critical for its representation in global climate models^{3,4}. Numerical simulations suggest that Arctic ice thickness varies primarily on decadal timescales^{3,5,6} owing to changes in wind and ocean stresses on the ice^{7–10}, but observations have been unable to provide a synoptic view of sea ice thickness, which is required to validate the model results^{3,6,9}. Here we use an eight-year time-series of Arctic ice thickness, derived from satellite

altimeter measurements of ice freeboard, to determine the mean thickness field and its variability from 65° N to 81.5° N. Our data reveal a high-frequency interannual variability in mean Arctic ice thickness that is dominated by changes in the amount of summer melt¹¹, rather than by changes in circulation. Our results suggest that a continued increase in melt season length would lead to further thinning of Arctic sea ice.

The prediction of future changes in Arctic sea ice, and consequent effects on the ocean¹² and atmosphere², relies on global climate models properly reproducing changes in ice thickness^{3,4,13}. Knowledge of ice thickness variability is also critical in determining whether observed changes¹⁴ are natural, or anthropogenic, in origin⁴. The sparseness of sea ice thickness observations means that current understanding of the regional, and interannual, variability of sea ice thickness is entirely based on numerical models of the Arctic^{6,9}. However, it is unclear from model results whether ice thickness is controlled mainly by changes in thermodynamic (radiative or thermal) forcing⁵, or by dynamic (ocean and wind stress) forcing⁷. The majority of Arctic Ocean models suggest that variability in Arctic ice thickness occurs on decadal timescales^{5,6,9}, and is caused mainly by dynamic forcing^{6–8}. Simulations of Arctic ice cover covering the past four decades have been used to argue that observed thin ice^{14–17} during the 1990s was a result of changes in atmospheric^{6,7,10,17} or oceanic^{8,18} circulation. However, numerical simulations of ice thickness are undermined by uncertainties in the representation of physical processes⁹, and by differences in methods used to couple the ice, ocean and atmosphere¹², resulting in significant discrepancies between model simulations of ice thickness evolution¹⁴. The lack of continuous large-scale thickness measurements means that conclusions drawn from numerical simulations regarding the variability of Arctic sea ice thickness, and the processes that control it, remain untested^{3,12}.

We use newly developed techniques to obtain ice thickness from satellite estimates of ice freeboard over the 8-yr period 1993–2001 (Fig. 1). The region of coverage (ROC) extends to 81.5° N, covering an average area of $3.08 \times 10^6 \text{ km}^2$, or more than half of the permanent sea ice cover. The data cover the entire circumference of the Arctic Ocean, including the Beaufort, Chukchi, East Siberian, Kara, Laptev, Barents and Greenland seas. We use measurements from the 13.8-GHz radar altimeters carried on the ERS-1 and ERS-2 satellites. By analysing individual echoes, we distinguish those originating from consolidated first and multi-year ice floes from those due to leads, open water and new ice. Corrections for orbits, tides, and atmospheric delay are applied to the radar data to obtain the elevation of ice floes and open water or new ice¹⁹. The elevation of the ice above the water surface is then obtained by subtracting the sea surface elevation, determined from open water measurements.

To deduce the ice thickness from ice elevation, the source of the echoes scattered from snow-covered sea ice must be determined. Laboratory experiments show that, under dry cold snow conditions, a normal-incidence 13.4-GHz radar reflection from snow-covered sea ice originates at the snow–ice interface²⁰. The ERS radar altimeter measurements of ice elevation therefore provide the level of the snow–ice interface above the water level—that is, the ice freeboard. We convert the ice freeboard measurements to ice thickness by assuming hydrostatic equilibrium, and then using fixed densities of ice (915.1 kg m^{-3}) and sea water ($1,023.9 \text{ kg m}^{-3}$)²¹ and a monthly climatology of snow depth and density²². The estimated uncertainty in ice and water density, of $\pm 5 \text{ kg m}^{-3}$ and $\pm 0.5 \text{ kg m}^{-3}$ respectively²¹, results in an uncertainty of $\pm 11 \text{ cm}$ for our mean thickness. Interannual variability in snow loading, estimated²³ to be between 2 and 3 cm, results in a further uncertainty of 6 to 9 cm in our ice thickness estimates. Figure 2 compares ERS thickness estimates with those derived from near-coincident submarine draught measurements¹⁵. A linear least-squares fit, weighted by the estimated measurement, snow loading and ice/water density uncertainties, shows that the correlation between the altimeter and

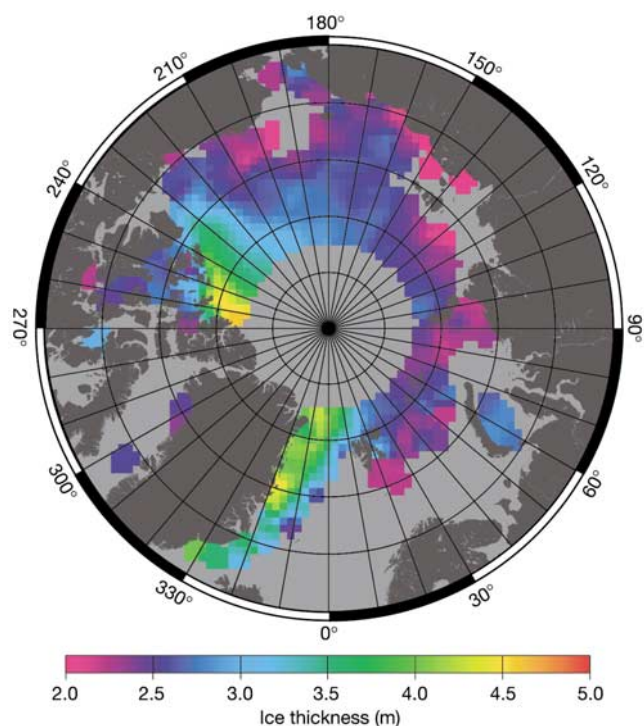


Figure 1 Average winter (October to March) Arctic sea ice thickness from October 1993 to March 2001 from satellite altimeter measurements of ice freeboard. Data are not available in the marginal ice zone, or above the ERS latitudinal limit of 81.5° N. Ice freeboard is converted to thickness using fixed ice, snow and water densities and regional monthly snow depth²². The mean thickness excludes thin ice (less than 0.5–1 m) and open water.

submarine estimates is significant at the 99.9% level using a χ^2 goodness-of-fit test.

The altimeter thickness estimates exclude areas of thin ice (less than 0.5 to 1 m) and open water. Using submarine draught data, gathered during different years, we calculate that the fraction of thin ice and open water ranges from $20.0 \pm 8.2\%$ in September (4 cruises) and October (1 cruise), to $3.2 \pm 1.4\%$ in March (2 cruises) and early April (5 cruises). Published submarine analyses show that rapid thermodynamic growth of thinner ice reduces the fraction of thin ice and open water from 20% to 10% between September and October²⁴, and further to 3% by April²⁵. Estimates from infrared satellite imagery show a reduction in thin ice fraction from 33% in September to 13% in October, and further to between 5 and 7% from January to April²⁶. The estimates differ from those obtained from submarines owing to the lower accuracy of the infrared technique and because the infrared data are from only a single winter season. Taking the average of the submarine estimates of thin ice and open water fraction in October (10%) and March (3%), we estimate that the altimeter data exclude an average of 6.5% of the ice cover during winter. We calculate, using the submarine data, that the consequent bias in mean winter thickness is +18 cm. The standard deviation of the bias, due to the interannual variability of thin ice and open water, and to sampling differences between different submarine cruises, ranges from 15 cm in September and October, to 5 cm in March and April. We therefore estimate that the maximum contribution of the thin ice and open water to the standard deviation in mean winter ice thickness is 10 cm.

We obtain a mean winter ice thickness over the ROC (Fig. 1) of 2.73 m. The thickest ice is observed adjacent to the Canadian Archipelago and in the Fram Strait, as observed in previously

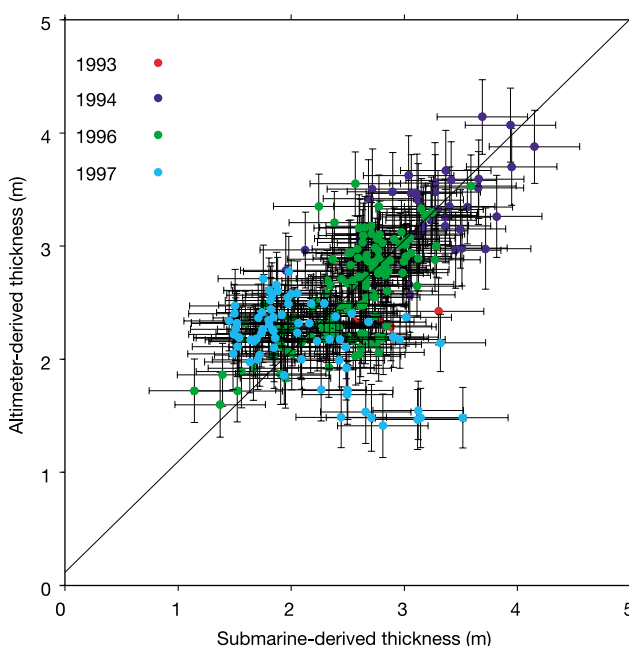


Figure 2 Comparison between satellite altimeter- and submarine-derived ice thickness in the Beaufort Sea during the 1990s. Submarine thicknesses are shown for each of the 50-km segments gathered during the four missions during the 1990s. Altimeter thickness estimates are generated from observations within 15 days and 100 km of the submarine draught sections. The submarine thicknesses exclude thin ice (<0.5 m) and open water, because (owing to difficulties in discriminating thin ice from open water) progressively more altimeter data are excluded once the ice thickness falls below 1 m. Error bars show uncertainties in altimeter thickness due to measurement errors and snow depth variability and an error in submarine thickness of 0.4 m, obtained by scaling the 0.3-m estimate of draught error¹⁵ and adding the uncertainty in the snow depth used to convert to thickness. A linear least-squares fit (shown) yields a slope of 0.978 ± 0.082 and intercept 0.117 ± 0.207 m, with a χ^2 probability $Q = 0.999$. We do not account for the different spatial sampling of the two techniques, which may result in additional scatter between the two data sets.

published climatologies based on sparse submarine observations²⁷. In the southern Beaufort Sea we observe an ice thickness of 2.5 m. This is thinner than the average submarine-observed draught of 3.7 m in 1976²⁷, but is consistent with field and submarine measurements during the 1990s^{15,17}. No submarine climatology exists for the eastern sector of the ROC, but the average thickness that we find between 60° E and 150° E (2.4 m) lies within the range of sparse *in situ* thickness measurements made in the same region during the mid-1990s (1.8 to 2.8 m; ref. 28). The regional distribution of ice thickness that we obtain is, therefore, similar to submarine climatologies, but consistent with thinner ice in the Western Arctic during the 1990s¹⁷.

We now examine the interannual variability in the average Arctic winter ice thickness over the ROC. We removed the mean thickness (Fig. 1) from individual measurements and, after applying a seasonal correction, averaged the residual thickness over each winter (October to March) to generate a time series of annual ice thickness anomalies (Fig. 3a). The average winter sea ice thickness over the ROC, excluding thin ice and open water, has a standard deviation of 24.5 cm, or 9% of the average, during the 8-yr period. This compares with a variability of 6% in ice mass in both Arctic⁵ and global coupled climate models^{3,29}, over periods of 50 yr or more. The data show that the observed variability in ice mass is 50% greater than predicted by models, and probably more, as our measure excludes variability that occurs over timescales of longer than a

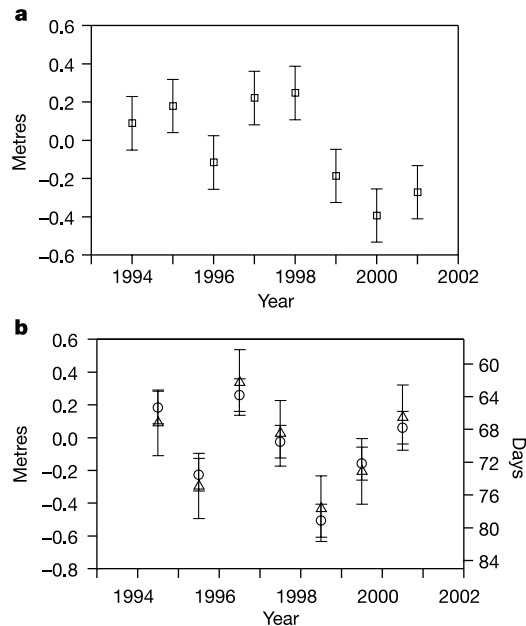


Figure 3 Time series of ice thickness anomalies and winter–winter change in thickness, compared with summer melt season length. **a**, Mean winter (October to March) ice thickness anomaly over the ROC for the period 1993–2001. The data show a spatial average of anomalies in ice thickness, obtained by subtracting the mean field (Fig. 1) from individual grids of average winter ice thickness. **b**, Changes in ice thickness between consecutive winters (circles) and melt season length (triangles) during the intervening summer period, derived from passive microwave observations¹¹. The correlation between ice thickness change and melt season length is $R^2 = 0.926$, showing that, during the period of our observations, the variability of mean Arctic sea ice thickness was controlled almost entirely by changes in thermodynamic forcing.

decade. The interannual variability in thickness compares with a variability in mean annual ice extent of 1.7% during the same period. This undermines the conclusion, from numerical models⁹, that changes in ice thickness occur on much longer timescales than changes in ice extent. The high-frequency variability shows that trends in ice thickness derived over periods of less than a decade¹⁵, or from a single pair of submarine cruises¹⁶, are susceptible to undersampling of the interannual variability in ice thickness. In summary, the observations show an interannual variability in ice thickness at higher frequency, and of greater amplitude, than simulated by regional Arctic models.

The mismatch between the observed variability and that predicted by models led us to investigate the origin of this variability. We compared the change in winter ice thickness with the length of intervening melt season, determined using satellite passive microwave radiometry¹¹ (Fig. 3b), by averaging all available data across the Arctic. Despite the fact that the spatial overlap between the two data sets is variable, we find a significant ($R^2 = 0.924$) correlation between the change in the altimeter-derived thickness between consecutive winters, and the melt season length during the intervening summer. Using a Monte Carlo simulation, we have calculated the change to the correlation that might occur if the altimeter estimates included thin ice and open water by adding a random component, with a standard deviation of 10 cm, to our average winter thickness estimates. Assuming a worst-case scenario, where the contribution of thin ice and open water to the change in mean winter ice thickness is uncorrelated with either the melt season length, or with the change in the altimeter thickness, the correlation is still significant ($R^2 = 0.745$). The regression implies that an

increase in the melt season length by one day results in an extra 4.9 cm of summer ice melt. *In situ* measurements obtained during 1997 at one location in the Beaufort Sea suggest typical surface melt rates³⁰ of 1.7 ± 0.5 cm per day. The difference between these figures may be due to varying melt rates across the Arctic, or to related changes in bottom melt, which continues beyond surface freeze-up³⁰. The observed dominant control of summer melt on the interannual variability of mean ice thickness (Fig. 3b) is in sharp contrast with the majority of models, which suggest that ice thickness variability in the Arctic Ocean is controlled mainly by wind and ocean forcing^{6–8,10}. It had also been speculated^{7,14} that reversal of the Arctic circulation in 1997 might lead to a thickening of the Arctic pack during the late 1990s. Instead, our data show a continued thinning of Arctic ice beyond 1998.

Our results show that errors are present in current simulations of Arctic sea ice, either in the radiative forcing, or in the parameterization of the effect of surface melt on the absorption of shortwave radiation. The observed variability of Arctic sea ice thickness, which shows that the sea ice mass can change by up to 16% within one year, contrasts with the concept of a slowly dwindling ice pack, produced by greenhouse warming¹³. This variability must be taken into account when determining the significance of trends derived from intermittent submarine measurements of ice draught. Although changes in Arctic circulation patterns may alter the distribution of ice thickness in the Arctic, we conclude that a continuation of the previously observed increase in melt season length¹¹ will lead to further overall thinning of Arctic sea ice. Until models properly reproduce the observed high-frequency, and thermodynamically driven, variability in sea ice thickness, simulations of both recent, and future, changes in Arctic ice cover will be open to question. □

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Neuroanatomy of flying reptiles and implications for flight, posture and behaviour

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Comparison of birds and pterosaurs, the two archosaurian flyers, sheds light on adaptation to an aerial lifestyle. The neurological basis of control holds particular interest in that flight demands on sensory integration, equilibrium, and muscular coordination are acute^{1–8}. Here we compare the brain and vestibular apparatus in two pterosaurs based on high-resolution computed tomographic (CT) scans from which we constructed digital endocasts. Although general neural organization resembles birds, pterosaurs had smaller brains relative to body mass than do birds. This difference probably has more to do with phylogeny than flight, in that birds evolved from nonavian theropods that had already established trends for greater encephalization^{5,9}. Orientation of the osseous labyrinth relative to the long axis of the skull was different in these two pterosaur species, suggesting very different head postures and reflecting differing behaviours. Their enlarged semicircular canals reflect a highly refined organ of equilibrium, which is concordant with pterosaurs being visually based, aerial predators. Their enormous cerebellar floccular lobes may suggest neural integration of extensive sensory information from the wing, further enhancing eye- and neck-based reflex mechanisms for stabilizing gaze.

The first vertebrate fliers were pterosaurs, an exclusively Mesozoic group that most workers¹⁰ regard as close relatives of Dinosauria within Archosauria (Fig. 1a). Pterosaurs were lightly built,

and their fossils are rare and often badly crushed. Virtual endocasts derived from CT scans of nearly complete skulls of two pterosaurs (Fig. 1)—the more basal *Rhamphorhynchus* and the pterodactyloid *Anhanguera*—are the most complete to date (Fig. 2; see Methods

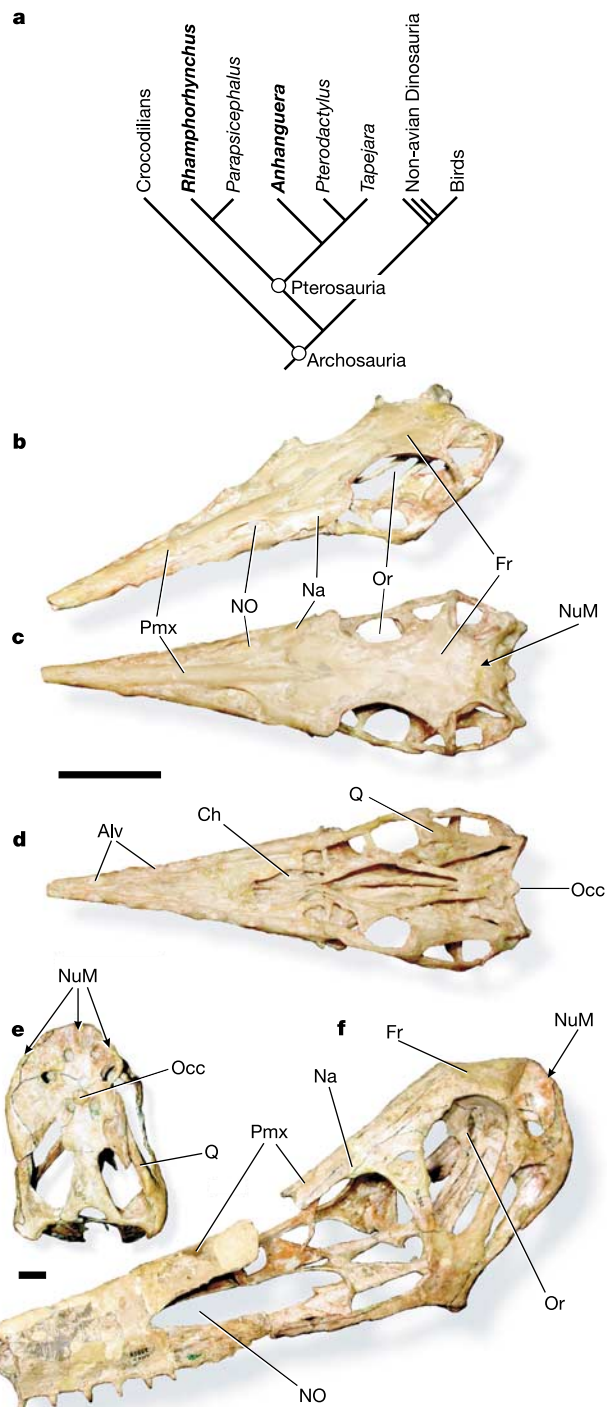


Figure 1 Relationships and skulls of pterosaur taxa. **a**, Cladogram of taxa mentioned in text and including pterosaurs for which endocast data are available. Topology based on ref. 19. **b–d**, *Rhamphorhynchus muensteri* (CM 11434, Jurassic, Germany) in left rostradorsolateral (**b**), dorsal (**c**), and ventral (**d**) views. **e, f**, *Anhanguera santanae* (AMNH 25555, Cretaceous, Brazil) in caudal (**e**) and left rostradorsolateral (**f**) views. Scale bar equals 20 mm. Alv, alveoli; Ch, choana; Fr, frontal; Na, nasal; NO, narial opening (confluent with antorbital fenestra in *Anhanguera*); NuM, area of attachment of nuchal (neck) musculature; Occ, occipital condyle; Or, orbit; Pmx, premaxilla; Q, quadrate.

and Supplementary Information). They confirm some previous findings of birdlike attributes^{1–8}: expansion of the cerebrum and cerebellum, displacing the enlarged optic tecta (lobes) ventrolaterally; small olfactory areas; and enlarged flocculi (cerebellar auricles) (Fig. 2). Despite these structural similarities, the brains of *Rhamphorhynchus* and *Anhanguera*, relative to body mass, do not fall within the range of extant birds, although they were enlarged relative to extant nonavian reptiles^{4,5,11} (Fig. 3; see Methods). Moreover, comparisons of total brain mass do not reveal differences in relative size of brain components (and hence underlying neural organization). For example, the enormous flocculi of pterosaurs probably outweighed the optic tecta, whereas the reverse is certainly true in birds.

Nevertheless, pterosaurs do possess a number of avian neuro-anatomical traits that may well be associated with the sensory and coordination functions necessary for flight. Jerison⁴ suggested that avian brains were relatively larger than those of pterosaurs because birds evolved in the environmentally complex and neurologically challenging arboreal habitat that required greater neural processing and hence greater mass. That may be true, but another factor is that birds and pterosaurs had different phylogenetic starting points: pterosaurs evolved from relatively very small-brained basal archosaurs, whereas birds evolved from theropod dinosaurs that had already initiated a substantial trend of brain expansion^{5,9}.

The virtual endocasts include the semicircular canals (Fig. 2), which had previously been only partially known for one pterosaur, *Parapsicephalus*¹. The entire osseous labyrinth is preserved bilaterally in *Anhanguera* and the large majority of it is preserved in *Rhamphorhynchus*. The semicircular canal system is greatly expanded, with the long canals encircling the flocculus. Its general arrangement closely resembles that of birds and some other dinosaurs⁵, but, whereas it is relatively modest in these groups, the

vestibular apparatus is relatively much larger in the pterosaurs. The well preserved osseous labyrinths in *Rhamphorhynchus* and *Anhanguera* provide an opportunity to test behavioural hypotheses of head orientation and posture. Researchers tend to reconstruct the head orientation of extinct animals with the skull's long axis (often the jawline) horizontal. Animals, of course, adopt a variety of head postures. There is a rich and taxonomically diverse literature supporting a robust empirical relationship between the planar elevation of the lateral semicircular canal and preferred head orientation^{12–16}. Determining 'preferred' head posture may seem problematic at first, but most vertebrates adopt a very stereotyped 'alert' posture^{13,17} and, moreover, retain this posture through a variety of behaviours¹⁵. Most studies agree that this preferred head orientation involves maintaining the lateral semicircular canal approximately level with the horizon (that is, 0° inclination) or elevated slightly in the front (5–10° inclination)^{12–16}.

Applying a conservative 5° inclination to pterosaurs shows that the long axis of the skull of *Rhamphorhynchus* indeed has a more or less horizontal orientation, as is typically portrayed⁶. However, bringing the lateral semicircular canal of *Anhanguera* to a position of 5° inclination results in the skull's long axis being strongly downturned (Fig. 4c). This dramatically different posture impacts on behavioural hypotheses relating to feeding and locomotion, perhaps allowing lateral scanning movements of the head (that is, in the plane of the lateral canals) to operate with optimal sensitivity¹³ or perhaps even simply allowing for a less obstructed view and greater overlap of the visual fields (binocular vision; Fig. 4e). Differences in head orientation may correlate with differences in body posture during terrestrial quadrupedal locomotion in that *Rhamphorhynchus*, with its relatively shorter forelimbs, must have adopted a more horizontal trunk, whereas *Anhanguera* had longer forelimbs and so had a more upright body posture^{6,18,19}, which in turn required a compensatory down-turning of the head. The aerody-

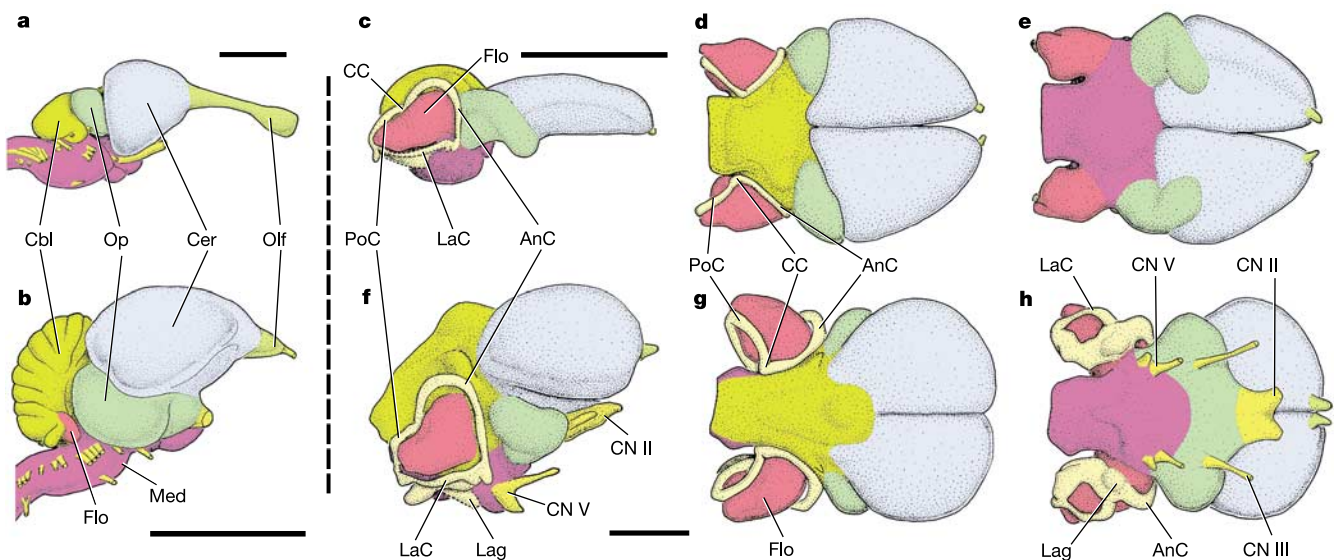


Figure 2 Endocasts and labyrinths of pterosaurs compared to brains of extant archosaurs. **a**, Brain of the crocodilian archosaur *Alligator mississippiensis* in right lateral view. **b**, Brain of the avian archosaur *Columba livia* (pigeon) in right lateral view. **c–e**, Endocast and osseous labyrinth of *Rhamphorhynchus muensteri* in right lateral view (**c**), dorsal (**d**), and ventral (**e**) views. **f–h**, Same of *Anhanguera santanae* in right lateral (**f**), dorsal (**g**), and ventral (**h**) views. Major areas of the brain are labelled for the alligator and pigeon, and the corresponding regions of the endocasts are given the same colour. The lagenar (cochlear) regions of both pterosaurs and some parts of the lateral semicircular canal of *Rhamphorhynchus* were not included in the virtual endocasts but were reconstructed from

the raw slice data. Some details of the ventral portion of the virtual endocast of *Rhamphorhynchus* were not well enough preserved to allow unequivocal reconstruction. **a** and **b** modified from originals^{29,30}. Scale bars equal 10 mm. AnC, anterior semicircular canal; Cbl, cerebellum; CC, crus communis; Cer, cerebrum; CN II, cranial nerve II (optic nerve); CN III, cranial nerve III (oculomotor nerve); CN V, cranial nerve V (trigeminal nerve); Flo, flocculus (cerebellar auricle); LaC, lateral (horizontal) semicircular canal; Lag, lagena (cochlea); Med, Medulla; Olf, olfactory lobe (bulb); Op, optic lobe (tectum); PoC, posterior semicircular canal.

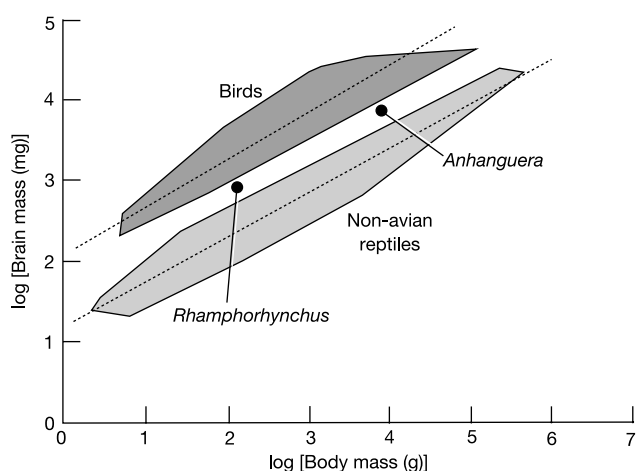


Figure 3 Relative brain size in pterosaurs compared to birds and other reptiles. Brain mass (using endocast mass as a proxy) in *Rhamphorhynchus muensteri* and *Anhanguera santanae* is relatively large in comparison to similarly sized reptiles, but does not fall within the range of extant birds. Data for taxa other than the pterosaurs derive from Hurlbert²⁸; see Methods for details and equations.

namic effects of a declined versus a perhaps more streamlined horizontal head orientation have not been fully assessed, but merit consideration¹⁸, particularly given the great length of the skull of *Anhanguera* and the fact that the bill bears a crest. It is worth noting that the occiput bears an extensive and robust attachment surface for neck musculature, and the cervical vertebrae are similarly stout (Fig. 1e–f). Thus, the head and neck appear to have been well-adapted to resist the large sagittal bending moments induced by aerodynamic forces in the declined head posture model (Fig. 4c). *Rhamphorhynchus*, by comparison, appears to have had only

modest neck musculature (Fig. 1c). Also, whereas the skull of *Rhamphorhynchus* is broad dorsally, the skull of *Anhanguera* is narrow and more triangular in cross-section (Fig. 4e), such that a declined head posture may incur less drag than might be suggested by a lateral view¹⁸.

The most striking and divergent aspect of pterosaur neuroanatomy is the space devoted to the sense of equilibrium. Comparing lateral views of the brains and semicircular canals of the two pterosaurs to that of a bird (for example, pigeon) shows that the labyrinth of pterosaurs occupies fully twice as much area, relatively, as the bird. This expansion in pterosaurs is all the more remarkable considering that birds themselves, relative to mammals, have enlarged labyrinths²⁰. The avian expansion has generally been associated with flight and the attendant requirements for balance and control in a three-dimensional aerial environment²¹. Semicircular canals sense angular acceleration (that is, head rotation), and canals with larger radii have increased sensitivity¹⁶. Moreover, animals (for example, birds and primates) with larger canals have been shown to be generally more aerobic or acrobatic^{16,21,22}. Thus, labyrinth expansion in pterosaurs is to be expected, although perhaps not to the extent observed here.

The great enlargement of the flocculus in pterosaurs, on the other hand, although reported previously^{2,5}, has not been widely appreciated. In extant nonavian reptiles, the flocculus is inconspicuous, whereas in birds and many other dinosaurs, the flocculus is larger and housed in a small bony recess. In *Rhamphorhynchus* and *Anhanguera*, however, the flocculus is larger than the optic tectum, forming a prominent lobe projecting from the caudolateral corner of the cerebellum (Fig. 2). The virtual endocasts allow quantification of relative floccular size. In *Rhamphorhynchus* and *Anhanguera*, the flocculi occupy about 7.5% of total brain mass, whereas in birds their relative mass is much less (1–2%). No other vertebrate group has so expanded the flocculus. In fact, the enlarged semicircular canals could be an epiphenomenon of primary floccular enlargement in that the canals are apparently

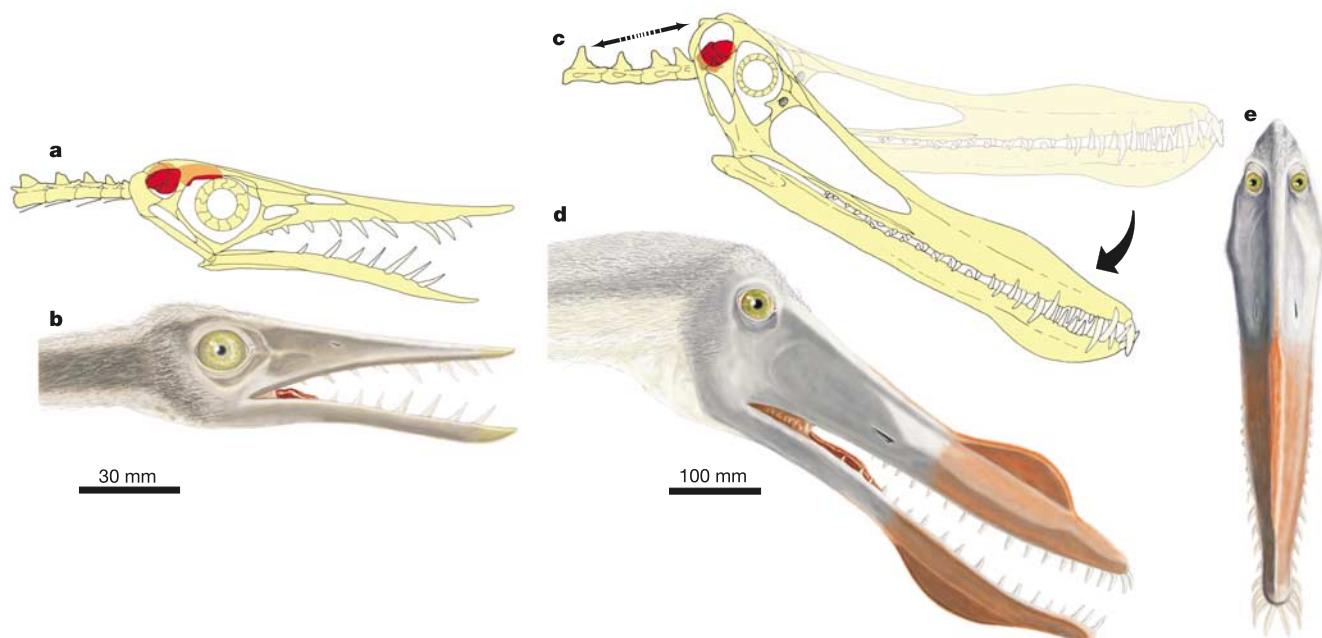


Figure 4 Skull and head postures of pterosaurs. **a, b**, *Rhamphorhynchus muensteri* skull (**a**) and restored head (**b**) in side view. **c–e**, *Anhanguera santanae* skull (**c**) and restored head in side (**d**) and front (**e**) views. Brains (endocasts) are indicated in red in **a** and **c**. In **c**, the declination of the skull (curved arrow) from horizontal (pale) reflects a head

posture with the lateral canal inclined 5° above horizontal. The double-headed arrow in **c** indicates the well-developed neck muscles that would have prevented excessive sagittal deflection of the head caused by aerodynamic drag. **a** and **c** modified from originals^{25,26}.

constrained to encircle the flocculus.

The flocculus has important connections with the vestibular system (via brainstem nuclei), the eye muscles (which are approximately coplanar with the canals), and, in some taxa, the neck muscles^{23,24}. This circuitry is best understood in the context of the vestibulo-ocular (VOR) and vestibulocollic (VCR) reflexes whereby coordination of head, eye and neck movements ensures stabilization of an image on the retina, preventing blurring^{16,24}. These reflexes allow a cheetah or a hawk to maintain a rock-steady gaze as it pursues its prey. Because some of the processing takes place in the flocculus, it would seem that pterosaurs devoted (perhaps even diverted) considerable neural resources to the integration of these gaze-stabilization mechanisms. Enhancement of such mechanisms seems reasonable in these two visually oriented pterosaurs given their apparent foraging style of aerial fish-eating^{6,25,26}.

However, there is no reason to believe that pterosaurs were more agile or aerobatic than birds (including avian aerial piscivores), and hence the size of the flocculus and semicircular canals remains enigmatic. Under the principle of proper mass (the amount of neural tissue in a structure is proportional to the amount of processing⁴), it would seem that the flocculus of pterosaurs was the site of neural processing unlike that seen in extant vertebrates, suggesting that an explanation should be sought among the unusual features of pterosaurs, such as the large, skin-covered flight membrane of the wing. In birds and mammals, the flocculus receives inputs carrying proprioceptive and cutaneous information (with a relay in the inferior olivary nucleus)²⁴. Thus, the pterosaur flocculus may have processed an unusually high volume of proprioceptive and somatosensory information associated with the wing membrane that stretched between the limbs, as well as with the limb joints themselves, consequently having a direct impact on the VOR/VCR and flight control. Support for enhanced proprioceptive input from pterosaur wings comes from the recent finding that the flight membrane incorporated muscle and tendon²⁷, which would have sent proprioceptive (muscle spindle) fibres back to the central nervous system, potentially for integration in the flocculus. Thus, the enlarged flocculus may be causally linked to the pterosaur integumentary wing membrane, with the wing providing to the flocculus potentially massive amounts of sensory data on attitude and body orientation, resulting in enhanced compensatory reflexes for maintaining the fixation of gaze upon a target. □

Methods

Imaging

Information on the brains of extinct organisms has traditionally come from naturally occurring endocasts of the brain cavity¹, latex endocasts^{7,8} made after the rock has been removed, and reconstructions of ground thin-sections of skulls⁴. We used a newer, noninvasive technique employing X-ray computed tomography (CT) to reconstruct a digital or 'virtual' endocast from the transverse CT slices of the brain cavity and vestibular apparatus. One skull each of *Rhamphorhynchus muensteri* (CM 11434; from the Upper Jurassic Solnhofen Lithographic Limestones of southern Germany) and *Anhangueria santanae* (AMNH 25555; from the Lower Cretaceous Santana formation of northeastern Brazil) was acid-prepared to remove surrounding matrix and then CT-scanned. *Rhamphorhynchus* was scanned along the coronal axis for a total of 476 slices, each slice 0.25-mm thick, with an interslice spacing of 0.2 mm (for a slice overlap of 0.05 mm). *Anhangueria* was scanned along the coronal axis for a total of 595 slices, each slice 0.50-mm thick with an interslice spacing of 0.45 mm (for a slice overlap of 0.05 mm). Raw slice data, reconstructed skulls, and virtual endocast animations are provided in the Supplementary Information.

Relative brain size calculations

Allometric scaling of brain mass (M_{Br}) and body mass (M_{Bd}) provides a means of comparing relative brain size. Jerison⁴ facilitated comparison by devising a simple metric, the encephalization quotient (EQ), which is a ratio of actual brain mass to the predicted brain mass (based on allometry) for the animal's reference group (for example, mammals, reptiles). We employ Hurlburt's²⁸ modifications of Jerison's method, such that for reptiles, $EQ = M_{Br}/(0.155(M_{Bd})^{0.55})$, whereas for birds, $EQ = M_{Br}/(0.117(M_{Bd})^{0.59})$. We use the avian equation to estimate the pterosaur EQs because pterosaur brains filled the endocranial cavity (as in birds, but unlike in reptiles). Mass estimation for *Rhamphorhynchus* (CM 11434) and *Anhangueria* (AMNH 25555) was complicated by lack of associated postcranial skeletons. We obtained body masses from comparably sized complete skeletons: *Rhamphorhynchus muensteri* (SMF R4128²⁵) and *Anhangueria piscator*

(NSN-PV 19892²⁶). Using a principal-components analysis method for estimating body mass¹⁸, M_{Bd} for *Rhamphorhynchus* is 136 g, and M_{Bd} for *Anhangueria* is 7,600 g. Brain volume (determined from CT) multiplied by density (1.036 g cm^{-3}) yields M_{Br} of 0.83 g for *Rhamphorhynchus* and 7.72 g for *Anhangueria*. EQ is 0.39 for *Rhamphorhynchus* and 0.34 for *Anhangueria*. Plotting log-transformed values on Hurlburt's²⁸ graph of brain versus body-mass shows that these pterosaurs fall between the reptile and avian polygons, with their EQs below those of birds (Fig. 3).

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Spontaneously emerging cortical representations of visual attributes

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Spontaneous cortical activity—ongoing activity in the absence of intentional sensory input—has been studied extensively¹, using methods ranging from EEG (electroencephalography)^{2–4}, through voltage sensitive dye imaging^{5–7}, down to recordings from single neurons^{8,9}. Ongoing cortical activity has been shown to play a critical role in development^{10–14}, and must also be essential for processing sensory perception, because it modulates stimulus-evoked activity^{5,15,16}, and is correlated with behaviour¹⁷. Yet its role in the processing of external information and its relationship to internal representations of sensory attributes remains unknown. Using voltage sensitive dye imaging, we previously established a close link between ongoing activity in the visual cortex of anaesthetized cats and the spontaneous firing of a single neuron⁶. Here we report that such activity encompasses a set of dynamically switching cortical states, many of which correspond closely to orientation maps. When such an orientation state emerged spontaneously, it spanned several hypercolumns and was often followed by a state corresponding to a proximal orientation. We suggest that dynamically switching cortical states could represent the brain's internal context, and therefore reflect or influence memory, perception and behaviour.

To determine the existence of spontaneously occurring states that correspond to cortical representations of orientations and characterize their dynamics, we chose to explore cat area 18, where most cells are selective for stimulus orientation, and therefore robust functional maps corresponding to different orientations are readily revealed. We used voltage sensitive dye imaging, which emphasizes synaptic membrane potential changes (similar to intracellular recordings from large populations of neurons^{18,19}). We recorded activity continuously in 30-s sessions (3,072 frames spaced 9.6 ms apart, covering a cortical area up to 4 × 7 mm) both in the presence and absence of stimulation (full field oriented gratings, see Methods for details). We used the evoked data to construct single-condition

and full-orientation maps, and used spatial correlation coefficients between single frames of ongoing activity and the evoked maps to evaluate similarity. Figure 1 illustrates the resemblance between a spontaneous single frame (Fig. 1b), its best correlated orientation map (Fig. 1a), and a single evoked frame (Fig. 1c). On average, the maximal correlation coefficient for evoked frames with any particular map was only $10 \pm 5\%$ higher than the maximal correlation coefficients seen for spontaneous frames (0.63 and 0.58 respectively in the example of Fig. 1). It is pertinent to note that the correlation coefficients between two maps obtained using the same stimulus, but in different recording sessions, usually ranged between 0.7 and 0.8.

To establish that such intrinsic orientation states occurred spontaneously much more frequently than expected by chance, we constructed control 'artificial orientation maps' (see Methods). We compared the distribution of correlation coefficients between spontaneous frames and the orientation maps (Fig. 2, red) with the corresponding distribution obtained using the control maps (Fig. 2, blue). Although both distributions were symmetrical around zero, the one computed with the real orientation maps was much wider. Specifically, whereas the maximal correlation coefficient with control patterns rarely exceeded 0.2 (less than 1% of the time), the corresponding values for the real orientation maps reached values as high as 0.6, with the mean maximal value across all hemispheres and imaging sessions being 0.5 ± 0.1 . Overall, the threshold for significant correlation ($P < 0.01$) was found to range between $|0.18|$ and $|0.22|$ using any of the control patterns. For subsequent analysis we conservatively set the threshold for significant correlation at $|0.25|$. Using this threshold, we found that states corresponding to orientation maps arise spontaneously about 20% of the time. Furthermore, we found that the amplitude of the most highly correlated spontaneous states was on average only 30% lower than the amplitude of the most highly correlated evoked single frames (see Supplementary Information S11 for additional information).

To characterize the distribution of spontaneous occurrences of different orientation states, we quantified the occurrence of spontaneous frames that were significantly correlated with each of the orientation maps. The obtained distribution was biased to states corresponding to one of the cardinal orientations (0° and 90°). An example from one hemisphere is shown in Fig. 3a. Overall, the maps corresponding to the two cardinal orientations appeared 20% more often than those corresponding to the two oblique ones (45° and 135°), but this dynamical bias for different cats was highly variable, ranging from 10% to 80% (see example from three extreme cases in Fig. 3b, green). Additionally, states corresponding to oblique maps emerged with smaller correlation coefficients than cardinal ones (Fig. 3b, blue). These results indicate a strong dynamical over-

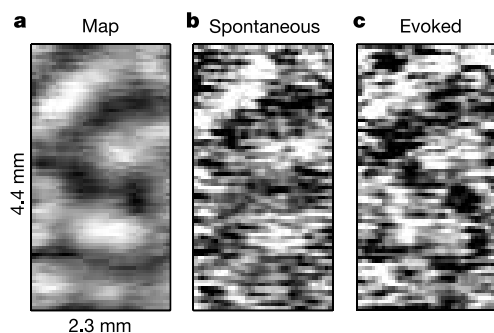


Figure 1 Comparing instantaneous patterns of spontaneous and evoked activity to the averaged functional map. **a**, The orientation map using full-field gratings of vertical orientation, obtained by averaging 165 frames (5 frames from each trial starting from 100 ms after stimulus onset, over 33 stimulus presentations). **b**, A map obtained in a single frame from a spontaneous recording session. **c**, A single frame from an evoked session using the same orientation as for the map. Amplitude was computed as described in Methods.

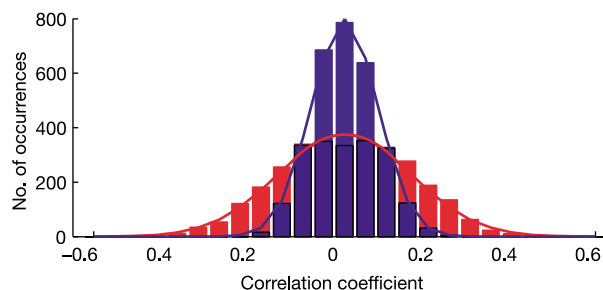


Figure 2 Spontaneously emerging orientation states. Red, example of the distribution of correlation coefficients between the horizontal map and spontaneous frames over an entire session. Blue, the same, using an inverted map. Each distribution was fitted to a gaussian to compute the significance level, $|0.25|$ for $P < 0.01$. The distribution was similar regardless of control maps used. Actual significance values were computed from the histograms resulting from correlations with all maps. Note that although low-pass filtering the frames and maps predictably increased the value of the correlation coefficients, the number of 'significant frames' remained similar, as the control correlation coefficients increased proportionally.

representation of the cardinal orientations, and are in agreement with recent observations of orientation anisotropies in area 18 of the cat visual cortex^{20,21}. Interestingly, in our voltage sensitive dye maps, we did not observe any consistent over-representation of the cortical areas corresponding to the cardinal orientations. This result is in agreement with some of the previous findings²² (unlike the large bias reported for the ferret^{23,24}). This finding is intriguing, underscoring the importance of exploring both function and dynamics.

To examine the extent of the cortical area spanned by a given spontaneously emerging cortical state, we divided evoked maps into two non-overlapping regions (illustrated in Fig. 3c), and examined how each region correlated with the corresponding area in each of the instantaneous frames of ongoing activity. We found that most of the time when the significance level was crossed, the two non-overlapping regions of the state emerged either simultaneously (Fig. 3d, plus signs) or in a close temporal proximity (circles). The results are illustrated by Supplementary Information SI2 (a movie) and additional examples are shown in Supplementary Information SI3.

Finally, in order to study the intrinsic states of spontaneous population activity and their dynamics without explicitly using any stimulus-derived orientation maps, we applied a well-known Kohonen map algorithm²⁵. We began with an initial set of 40 random templates (frames) located on a closed circle, onto which the spontaneous single frames are mapped by finding the template that is closest to each frame. A Kohonen algorithm was applied to gradually update the templates so that in the end they characterize the hidden low-dimensional order in high-dimensional population activity (see Methods). A Kohonen algorithm was chosen for the analysis of the recordings because it required only spontaneous data, and did not necessitate prior assumptions about the number of hidden states. Although the templates were learned exclusively from the spontaneous data without making any use of the stimulus-evoked orientation maps, many of the 'learned templates' (after the algorithm converged) exhibited strong similarity to the orientation maps obtained from the evoked data. Figure 4A (bottom traces) depicts the templates that had highest correlation coefficients with

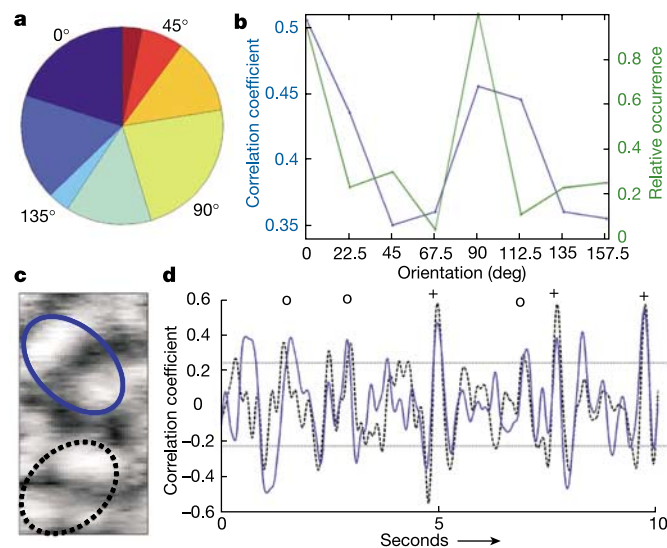


Figure 3 Dynamics of ongoing activity. **a**, Example of the distribution of occurrence of the different orientation states. **b**, The relationship between orientation and number of spontaneous occurrences of that orientation state (blue), or maximal correlation coefficient (green) (average from three hemispheres). **c**, Illustration of two non-overlapping patches on the map from Fig. 1a. **d**, Example of the time course of the correlation coefficients between each of the selected areas on an evoked map, and single frames from spontaneous activity sessions. Significance lines are plotted in accordance with Fig. 2a. Plus signs, synchronous occurrences of patches; circles, times of phase lag.

three of the orientation maps (top traces).

Because most of the final templates were good approximations to single-condition orientation maps, they could be used for estimating the orientation preference (OP) map. To this end, every template was assigned an orientation according to its position on the circle by selecting the template corresponding to horizontal orientation and adding an equal angle of 4.5° (180° divided by 40, the number of templates) to every subsequent template, and the OP of every pixel was then obtained by standard vectorial summation of the corresponding templates. The angle map calculated this way is shown in colour in Fig. 4B (right panel) together with the OP map obtained with visual stimulation (Fig. 4B, left panel). The similarity between these two maps is remarkable: the spontaneous activity alone contained most of the information about the OP maps. Figure 4Ca shows the orientation of the map that had the highest spatial correlation coefficient to each learned template. Note the systematic transitions between neighbouring orientations encountered as one moves along the set of templates. The values of the corresponding correlation coefficients are shown in Fig. 4Cb. The maximal correlation coefficients reached values as high as 0.8 for cardinal orientations, but were significantly lower for oblique orientations, in accordance with our findings of representation asymmetry.

Using the obtained templates, we analysed the dynamics of the spontaneous activity by looking at the temporal sequence of the templates onto which consecutive frames were mapped (Fig. 4Da, and movie in Supplementary Information SI4). Long epochs of smooth transitions between neighbouring templates were clearly present in the data. To check that these long epochs could not be explained merely by temporal correlations in the population activity (that is, the fact that spontaneous single frames have a finite rate of change), we performed a control analysis by generating artificial templates that had spatial structure and mutual cross-correlations similar to those of the original templates (see Methods). Using these synthetic templates indeed resulted in

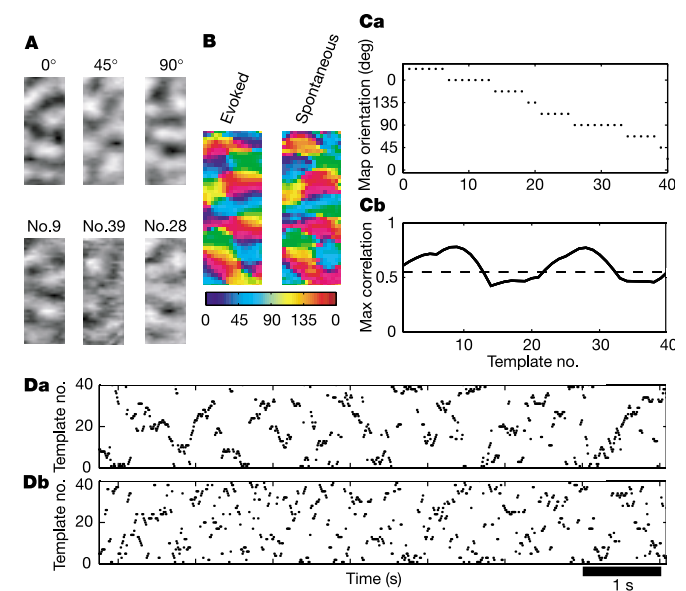


Figure 4 Spontaneous states revealed using a Kohonen algorithm. **A**, Top, three single-condition orientation maps. Bottom, three Kohonen templates best correlated with those maps. **B**, Left, evoked orientation map. Right, full orientation map computed using the 40 Kohonen templates. **Ca**, Orientation of the map that is maximally correlated to each Kohonen template. **Cb**, Correlation coefficient between each template and the corresponding orientation map. The dashed line indicates the significance level ($P < 0.01$) obtained with control synthetic templates (see Methods). **Da**, Position of a best-matching Kohonen template as a function of time. **Db**, Position of best-matching template from a set of control templates.

significantly shorter transitions (Fig. 4Db). The average transition for the experimental maps was twice as long as for the control (82 ms and 40 ms, respectively) and this difference was highly significant (t -test, $P < 10^{-16}$). The modelling of the geometry and dynamics of cortical states is further discussed in Supplementary Information SI5).

To conclude, we have shown that ongoing activity encompasses a set of dynamically switching cortical states, several of which correspond to cortical representations of orientations. The hypothesis that ongoing activity in the awake animal also contains a set of states is currently being tested. Because we do not usually perceive switching orientations while our eyes are closed, it is conceivable that states that are intrinsic to lower areas, but which are constantly interacting with incoming stimulation and feedback from higher areas²⁶, are not as persistent in duration or as spatially coherent in the awake animal as in the anaesthetized one. These findings support models of orientation maps that reflect intrinsic cortical states largely determined by intra-cortical connectivity^{27–29}, and suggest that dynamically switching cortical states could express the cortical way to prepare ‘default’ parameter values, which in turn reflect expectations about the sensory input. □

Methods

Imaging

We used optical imaging of voltage sensitive dyes combined with single unit recordings. The technicalities of the method are discussed extensively elsewhere^{6,7,18,30}. We used RH1691 to dye the cortex, and a high-speed camera to record the changes in the stained cortex. Data from nine hemispheres of six cats were collected and analysed (20–40 sessions per hemisphere both with and without stimulation). Each recording session lasted 30 s, and 3,072 frames 9.6 ms apart were obtained. For evoked sessions of continuous recording, over the course of the 30 s of recording, 33 stimuli of the same orientation were presented (trials). The stimuli consisted of full-field drifting gratings of high contrast lasting 200 ms, with an isoluminant 700-ms inter-stimulus interval using a grey screen. In each hemisphere, we used evoked activity to establish the basic set of states by obtaining eight single condition orientation maps at 22.5° steps. Maps in each evoked recording session were computed by averaging 165 frames; this was done by using the five frames occurring between 100 and 150 ms after stimulus onset, from each of the 33 stimuli presented over the course of a single session; $33 \times 5 = 165$). For ongoing activity recording sessions the animal's eyes were closed, and the room darkened (we found no difference when a uniform grey screen was used instead). Each recorded frame represented the instantaneous pattern of activity over a large patch of cortex (up to 4×7 mm in size; size of each pixel is $64 \times 64 \mu\text{m}$). The amplitude of each single frame was computed as the difference between the signal strength at the centres of the four most responsive patches (‘white patches’), and the four least responsive patches (‘black patches’). The locations of the patches were defined on the evoked maps, and for each frame we selected the map that most closely corresponded to that frame. We quantified the similarity between the patterns of any individual frame and each of the orientation maps by the correlation coefficient between the two. Note that the results in each recording session were entirely independent of stimulation history. The data was cleaned for breathing artefacts through high-pass temporal filtering above 0.6 Hz. Heart artefacts were removed by subtracting the average heart pattern for each pixel. All experiments were performed in strict accordance with institutional and NIH guidelines.

Kohonen maps

The Kohonen map algorithm was applied for a topologically ordered mapping from the space of recorded multidimensional images to a one-dimensional array of M nodes evenly distributed on a closed circle. With every node i , a template image m_i was associated. The randomly initiated templates were modified iteratively during training. Each data image $x(n)$ at a time bin n was compared with all the templates according to the euclidean distances in order to define the best-matching node (c): $c = \arg \min_i \|x - m_i\|$. The euclidean distance $\|x - m_i\|$ is a square root of a sum over all image pixels of square differences of signal intensities. The templates were then updated, such that every template was made to be slightly more similar to the current data image. The amplitude of the change for each template depends on its proximity to the best-matching node according to a neighbourhood function, $h(c - i)$: $m_i(n + 1) = m_i(n) + \alpha h(c - i)[x(n) - m_i(n)]$.

The neighbourhood function was chosen as a gaussian function of a position on the circle:

$$h(c - i) = \exp\left(-\frac{\{\min(|c - i|, M - |c - i|)\}^2}{2\sigma^2}\right)$$

For convergence it is necessary that the rate of change, α , gradually decreases during the training. We started with a value of α close to unity, and decreased it monotonically during the first training phase of 3,072 iteration steps, keeping it small (close to 0.01) afterwards. We also started with a fairly large σ , and decreased it as the proper ordering took place. The number of iterations was chosen such that each data frame is used several times (5–10). In order to construct the set of control templates, we first obtained a synthetic map by

convolution of a random image with a typical orientation preference map. The rest of the templates were formed by consequent shifts of the original synthetic map over a circular trajectory. The radius of the circle as well as the amplitudes of the shifts was chosen in such a way that resulting synthetic templates have similar cross-correlation structure to the ones obtained with the Kohonen algorithm. Significance level was calculated from a distribution of maximal correlations between orientation maps and the set of 1,000 different synthetic templates.

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Positioning of follicular dendritic cells within the spleen controls prion neuroinvasion

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Peripheral infection is the natural route of transmission in most prion diseases¹. Peripheral prion infection is followed by rapid prion replication in lymphoid organs, neuroinvasion² and progressive neurological disease. Both immune cells and nerves are involved in pathogenesis^{3,4}, but the mechanisms of prion transfer from the immune to the nervous system are unknown. Here we show that ablation of the chemokine receptor CXCR5 juxtaposes follicular dendritic cells (FDCs) to major splenic nerves, and accelerates the transfer of intraperitoneally administered prions into the spinal cord. Neuroinvasion velocity correlated exclusively with the relative locations of FDCs and nerves: transfer of CXCR5^{-/-} bone marrow to wild-type mice induced perineural FDCs and enhanced neuroinvasion, whereas reciprocal transfer to CXCR5^{-/-} mice abolished them and restored normal efficiency of neuroinvasion. Suppression of lymphotoxin signalling depleted FDCs, abolished splenic infectivity, and suppressed acceleration of pathogenesis in CXCR5^{-/-} mice. This suggests that prion neuroimmune transition occurs between FDCs and sympathetic nerves, and relative positioning of FDCs and nerves controls the efficiency of peripheral prion infection.

Lymphoid organs are early sites of prion accumulation and replication following intraperitoneal (i.p.) inoculation^{5,6}, and properly structured B-cell follicles are essential for peripheral pathogenesis⁷. B cells provide lymphotoxins and tumour necrosis factor, which contribute to maturation and maintenance of FDCs. B-cell ablation suppresses prion neuroinvasion^{3,8}, probably because B cells transmit lymphotoxin-dependent signals to FDCs⁹, which accumulate disease-associated prion protein (PrP^{Sc}) following scrapie infection¹⁰. Sympathectomy delays the onset of scrapie after i.p. inoculation of the infectious agent, whereas sympathetic hyperinnervation enhances splenic prion replication and neuroinvasion¹¹, suggesting that innervation of lymphoid organs is important to neuroinvasion. However, there is no physical synapse between FDCs and sympathetic nerve endings¹², and it is unclear how prions accomplish transfer from immune cells to the nervous system.

Here we have studied how the distance between FDCs and splenic nerves affects the velocity of neuroinvasion. FDC positioning was manipulated by ablation of the CXCR5 chemokine receptor, which directs lymphocytes towards specific microcompartments¹³. Histological analysis of prion-infected spleens revealed that B cells of wild-type (WT) mice accumulate at the periphery of white pulp follicles in discrete CD35⁺ and PNA⁺ clusters, whose periarteriolar sheaths consist predominantly of T cells (Fig. 1a and data not

shown). By contrast, splenic follicles of CXCR5^{-/-} mice contained an inner CD35⁺ and PNA⁺ B-cell cuff around central arterioles (Fig. 1a and data not shown) enclosed by concentric T- and B-cell sheaths. Mature FDCs (CD35⁺FDC-M1⁺) of CXCR5^{-/-} mice are germinal-centre-associated and immediately adjacent to central arterioles¹⁴, whereas in WT mice, FDCs are antipodal to follicle vessels (Fig. 1a, right panel).

Sympathetic nerve processes are tyrosine hydroxylase (TH)-positive, and mainly line splenic vessels¹⁵. Visualization of these nerves failed to reveal differences in density, distribution and branching patterns between WT and CXCR5^{-/-} spleens (Fig. 1b). However, the distance between CXCR5^{-/-} FDCs and splenic nerves was greatly reduced (Fig. 1b, upper panels). Total splenic innervation was quantitatively assessed by western blotting: The tyrosine hydroxylase contents of WT and CXCR5^{-/-} spleens were similar (Fig. 1c) and much lower than in K14NGF transgenic spleens¹⁶, which exhibit increased sympathetic innervation¹¹.

CXCR5^{-/-} mice were inoculated with serial dilutions of scrapie prions, and incubation times to terminal disease were measured. After intracranial (i.c.) challenge (3×10^5 or 3×10^2 LD₅₀ (50% intracerebral lethal dose) units), all CXCR5^{-/-} and WT mice developed scrapie with similar average incubation times (142 ± 4 days in CXCR5^{-/-} and 139 ± 6 days in WT mice exposed to 3×10^5 LD₅₀ units; 166 ± 4 days in CXCR5^{-/-} and 172 ± 8 days in WT mice exposed to 3×10^2 LD₅₀). Therefore, CXCR5 deficiency does not affect any aspect of prion propagation within the central nervous system (CNS). In both genotypes, i.p. challenge with as little as 3 log LD₅₀ resulted in 100% attack rate (Fig. 1d).

Maximal speed of pathogenesis after delivery of a large inoculum (6 log LD₅₀) was similar in CXCR5^{-/-} and WT mice (CXCR5^{-/-}, 202 ± 8 days; WT, 199 ± 6 days). Surprisingly, when mice were confronted with smaller inocula, incubation times increased dose-dependently in WT mice, but much less so in CXCR5^{-/-} mice (Fig. 1d). After exposure to 3 log LD₅₀, CXCR5^{-/-} mice developed scrapie by 215 ± 2 days post inoculation (d.p.i.), and WT mice only at 242 ± 8 d.p.i. Regression analysis indicated that latency was inversely proportional to the logarithm of the inoculum¹⁷: WT mice followed the formula $t_1 = 285 - 14 \log \text{LD}_{50}$ (where t_1 is incubation time; correlation coefficient $r = 0.99$; $P < 0.001$). Instead, CXCR5^{-/-} mice had a much flatter dose response ($t_1 = 228 - 4.5 \log \text{LD}_{50}$; $r = 0.98$; $P < 0.01$). By contrast, i.p. inoculation of *tga20* transgenic mice¹⁸, which overexpress PrP^C, yielded the relationship $t_1 = 192 - 15 \log \text{LD}_{50}$, with a slope virtually identical to that of WT mice (Fig. 1d). We conclude that in *tga20* mice pathogenesis was uniformly accelerated, whereas in CXCR5^{-/-} mice it became more and more efficient upon incremental reduction of the inoculum.

Terminal illness was unambiguously accompanied by clinical scrapie signs. Microanatomical distribution and intensity of spongiosis and gliosis were similar (not shown), and PrP^{Sc} accumulated to a similar extent in all brains (Fig. 2a) and spleens (not shown) of clinically sick CXCR5^{-/-} and WT mice.

Because B-cell ablation prevents lymphoid prion replication³, variations in B-cell subpopulations may alter prion susceptibility of CXCR5^{-/-} mice. However, the number and distribution of marginal zone (CD21^{high}CD23^{low}) and follicular B cells (CD21^{high}CD23^{high}) in spleens and lymph nodes, as well as their expression of adhesion molecules (β_1 -, β_2 -, α_4 -, $\alpha\text{L}\beta_2$ -integrin) was similar in CXCR5^{-/-} and WT mice (Fig. 2b). We found a 20-fold reduction of peritoneal B-1 cells in CXCR5^{-/-} mice (Fig. 2c), but this did not lead to reduced prion titres after i.p. inoculation in any organs tested at 40 and 80 d.p.i. (Fig. 2d, upper left panel), presumably because B-cell subsets directly involved in FDC maintenance were not affected.

Is the accelerated neuroinvasion in CXCR5^{-/-} mice due to enhanced lymphoid prion replication, or to more efficacious neuroimmune transfer of infectivity? The former possibility was

rejected by determining the kinetics of infectivity titres¹⁹ in spleens and lymph nodes of i.p. challenged mice (4 and 6 log LD₅₀) at two different time points (Fig. 2d, left panels). We then compared kinetics of infectivity after i.p. prion challenge (4 log LD₅₀) in spleens and thoracic spinal cords, to which the splanchnic nerves project^{11,20}. At 40 d.p.i. spleens were highly infectious (Fig. 2d, lower left panel), yet no prions were detectable in the spinal cords of either genotype (Fig. 2d, lower right). Infectivity rose to measurable levels at 60 d.p.i. in CXCR5^{-/-} spinal cords, whereas in WT mice traces of infectivity were first detected at 80 d.p.i. (Fig. 2d, lower right panel). Prion replication was unaltered in both the lymphoreticular and the central nervous compartment, so the precocious rise of infectivity in spinal cords suggests increased velocity of prion entry into the CNS.

Shortened incubation time (Fig. 1d) and accelerated prion build-up in CXCR5^{-/-} spinal cords (Fig. 2d) amounted congruently to approximately 30 days.

Because genetic or pharmacological ablation of lymphotoxin signalling dedifferentiates FDCs and protects mice from peripheral prion challenge^{7,9,21}, FDC hypertrophy in CXCR5^{-/-} mice may lead to acceleration of pathogenesis. Besides, as FDCs that form around central arterioles are germinal-centre-associated¹⁴, their frequency may vary between animals depending on infection load. However, inspection of splenic germinal centres in prion-infected mice revealed slightly more frequent FDC-M1⁺ networks in WT mice (36 networks per 35 mm²) than in CXCR5^{-/-} mice (29 networks per 35 mm²), whereas PNA⁺ areas were similar (WT, 23; CXCR5^{-/-}, 25

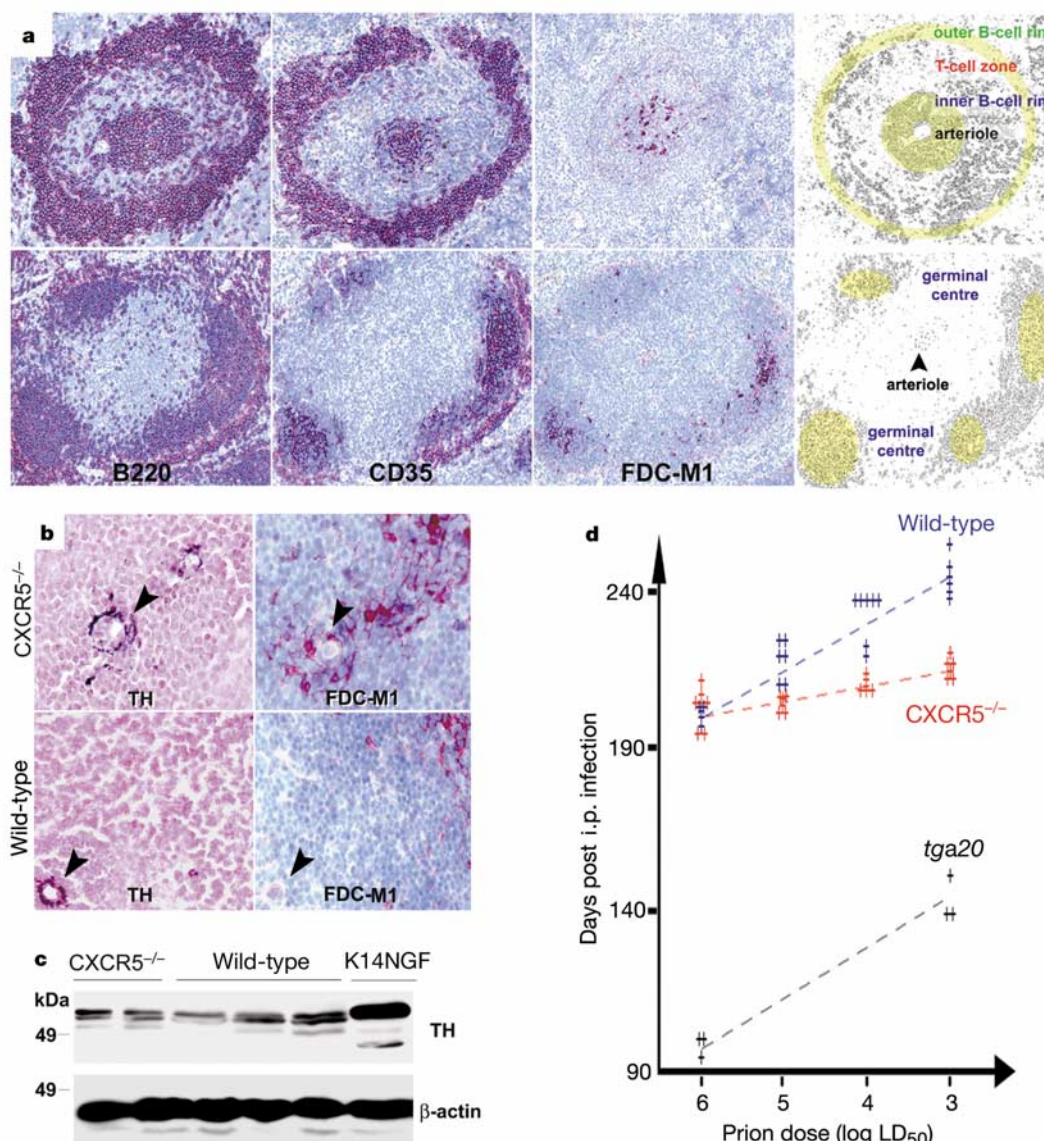


Figure 1 Neuroimmune anatomy of CXCR5^{-/-} and WT mice. **a**, Spleens of prion-infected CXCR5^{-/-} (top row) and WT mice (bottom row) were stained for B cells (B220), CD35 and FDCs (FDC-M1). CXCR5^{-/-} FDCs were atypically localized in the neighbourhood of vessels (see diagram on right of WT and CXCR5^{-/-} follicles). **b**, Microanatomical association between neural and immune elements of lymph follicles in CXCR5^{-/-} mice. Sympathetic nerves, visualized with anti-TH antibodies (top left), were in close vicinity to FDC-M1⁺ cells (top right) around splenic vessels, as indicated by arrowheads (×200). Instead, WT FDC-M1⁺ cells (bottom right) were localized in B-cell areas antipodal to TH⁺ peripheral nerves (bottom left, ×150). **c**, Tyrosine hydroxylase

content is similar in spleens of CXCR5^{-/-} and WT mice, but higher in K14NGF transgenic mice. Western blot analysis shows that the main TH bands (~55 kDa, top panel), are similar in adult CXCR5^{-/-} and WT mice, but stronger in K14NGF mice, consistently with increased sympathetic innervation. Membranes were reprobed with antibodies to β-actin (bottom panel). **d**, Enhanced neuroinvasion in CXCR5^{-/-} mice after peripheral prion challenge. Upon administration of 6 log LD₅₀ of prions, CXCR5^{-/-} (red) and WT (blue) mice developed scrapie with similar kinetics, whereas after 3–5 log LD₅₀ CXCR5^{-/-} mice became sick earlier than WT mice. tga20 (black crosses) mice progressed to disease more quickly than WT mice with all inocula.

positive areas per 35 mm²; $P > 0.05$) (Fig. 3a, left panel). Splenic FDC-M1⁺ networks were also slightly enlarged in WT mice ($44,042 \pm 13,977 \mu\text{m}^2$; $P < 0.01$) as compared with CXCR5^{-/-} mice ($32,303 \pm 8,912 \mu\text{m}^2$), whereas PNA-positive clusters were similar in WT ($14,499 \pm 8,393 \mu\text{m}^2$) and CXCR5^{-/-} mice ($15,785 \pm 8,768 \mu\text{m}^2$; $P > 0.05$). We also assessed the average area occupied by FDC-M1⁺ cells in spleen follicles of 12 CXCR5^{-/-} and eight WT mice before prion infection. Frequency of B-cell zones (4–8 mm⁻²) and follicle diameters (254–645 μm , defined by B220 immunostains) were similar in CXCR5^{-/-} and wild-type mice, and CXCR5^{-/-} FDC networks were smaller (Fig. 3a, right panel; $P < 0.05$). Therefore, enhanced pathogenesis was not due to FDC hypertrophy, or to hyperplasia of PNA⁺ clusters in infected mice.

Does CXCR5 deficiency enhance neuroinvasion by shifting prion replication to unrecognized FDC-independent compartments? This was addressed by treating CXCR5^{-/-} and WT mice i.p. with soluble lymphotoxin- β receptor (LT β R-Fc), or with pooled human IgG at -7, +14 and +28 d.p.i. Spleens were analysed at +38 d.p.i. (Fig. 3b)^{9,22}. LT β R-Fc, but not huIgG, efficiently depleted FDCs (Fig. 3a, b). B-cell follicles maintained a normal morphology; only occasional FDC-M1⁺ cells remained, possibly representing tingible body macrophages.

At 38 d.p.i., no PrP^{Sc} was detected by western blot analysis in WT and CXCR5^{-/-} spleens treated with LT β R-Fc, whereas huIgG-treated spleens exhibited conspicuous amounts of PrP^{Sc} (Fig. 3c). LT β R-Fc-treated spleens (both CXCR5^{-/-} and WT) contained $<1.5 \log \text{LD}_{50} \text{g}^{-1}$ prion infectivity, whereas titres in huIgG-treated spleens ranged from 4.2 to 4.6 (CXCR5^{-/-}) and from 4.1 to 4.5 (WT) $\log \text{LD}_{50} \text{g}^{-1}$. We conclude that LT β R-Fc treatment suppresses splenic PrP^{Sc} build-up and prion infectivity congruently, effectively, and independently of the CXCR5 genotype. Therefore, no FDC-independent prion replication occurs within CXCR5^{-/-} spleens.

If positioning of FDCs within follicles were indeed crucial for prion neuroinvasion, depletion of mature FDCs would prolong the incubation time of scrapie in peripherally challenged CXCR5^{-/-} mice. We inoculated CXCR5^{-/-} and WT mice with 3 $\log \text{LD}_{50}$ prions, and treated them with 100 μg LT β R-Fc (or human pooled IgG for control) i.p. at -7, +7, +14 d.p.i. CXCR5^{-/-} ($n = 3$) and WT ($n = 3$) mice treated with human IgG succumbed to scrapie with similar incubation times as untreated mice (CXCR5^{-/-}, 216 ± 1.7 days; WT, 245 ± 2.3 days). By contrast, CXCR5^{-/-} ($n = 3$) and WT mice ($n = 4$) treated with LT β R-Fc showed significant prolongation of survival time (>300 days, all mice

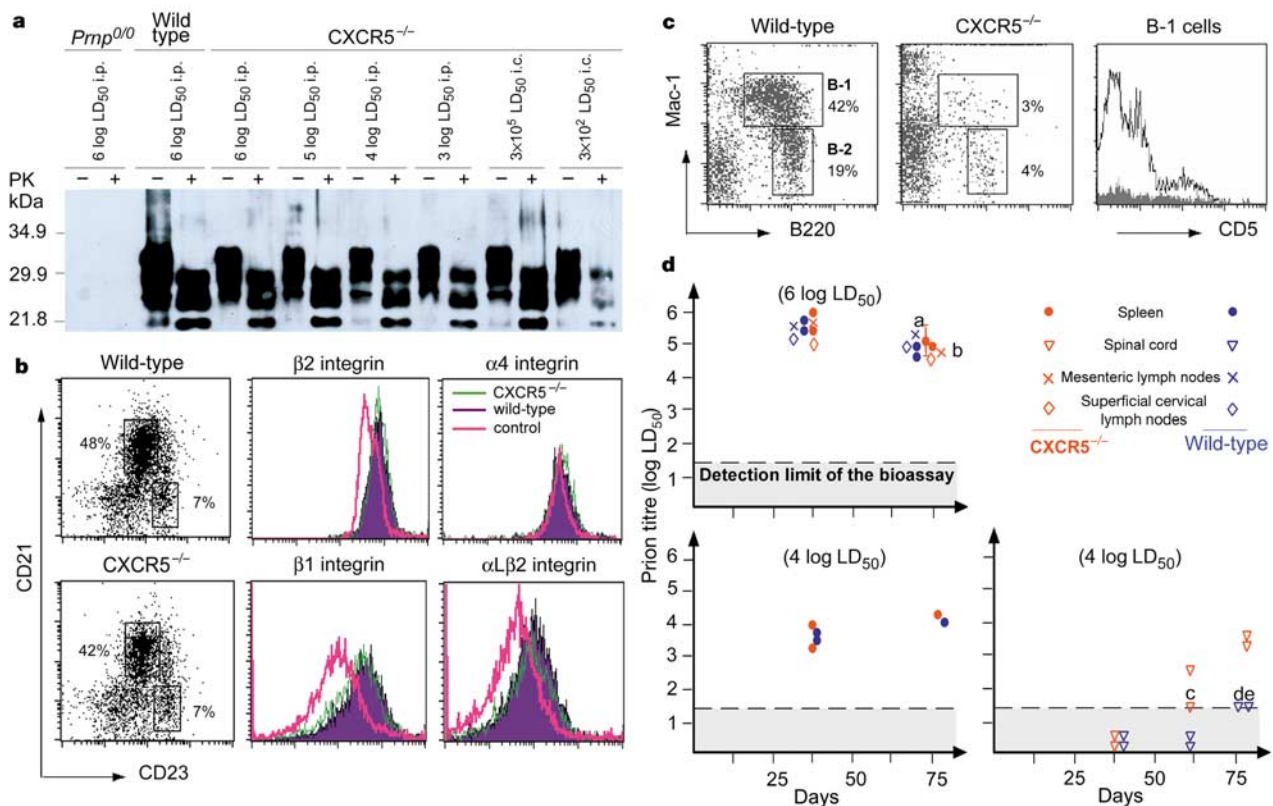


Figure 2 Kinetics of prion disease in CXCR5^{-/-} and WT mice. **a**, Western blotting of brain electrophoresed natively (–) or after digestion with PK (+) revealed large amounts of PK-resistant prion protein (PrP^{Sc}) in terminally scrapie-sick CXCR5^{-/-} and WT mice. **b**, Splenic B-cell compartments were numerically and phenotypically unaffected in CXCR5^{-/-} mice. Numbers indicate percentages of CD21^{high}CD23^{high} FB cells and CD21^{low}CD23^{high} MZB cells. Dot plots were gated on B220⁺ cells. Integrin expression of B220⁺CD21^{low}CD23^{high} cells (green, CXCR5^{-/-}; purple, WT). Integrin expression of B220⁺CD21^{high}CD23^{high} FB cells in WT mice, which corresponded to integrin expression of CXCR5^{-/-} mice, was measured as a control (pink). **c**, Flow cytometric analysis of Mac-1 and B220 expression by size-gated PerCP. Boxes demarcate Mac-1⁺B220^{hi} as B-1 and Mac-1⁺B220^{lo} as B-2 subsets (left and middle panel). CD5 expression on WT

(white) and CXCR5^{-/-} (grey) peritoneal B-1 cells (right panel). **d**, In CXCR5^{-/-} mice infectivity reached the spinal cord much earlier than in WT mice, whereas titres in spleens (red circles) and superficial cervical (red diamonds) and mesenteric (red crosses) lymph nodes of CXCR5^{-/-} mice were similar to their WT counterparts (blue symbols) at all time points. Each data point was calculated from the average survival of four *tga20* mice except for data points labelled a and b, which were derived from three *tga20* mice. Standard deviations were drawn only when exceeding $\pm 0.75 \log \text{LD}_{50}$. Symbols below the dashed line indicate titres below detection (0/4 indicator mice developed scrapie). If ≥ 1 indicator mice survived >180 d.p.i., or if the mean incubation time was >120 days, titres were regarded as close to the detection threshold, and symbols were placed on the dashed line. For these samples, attack rates and incubation times (in days) for data points c, d and e were as follows: c, 3/4 (87, 97, 105); d, 3/4 (78, 105, 105); e, 2/4 (90, 97).

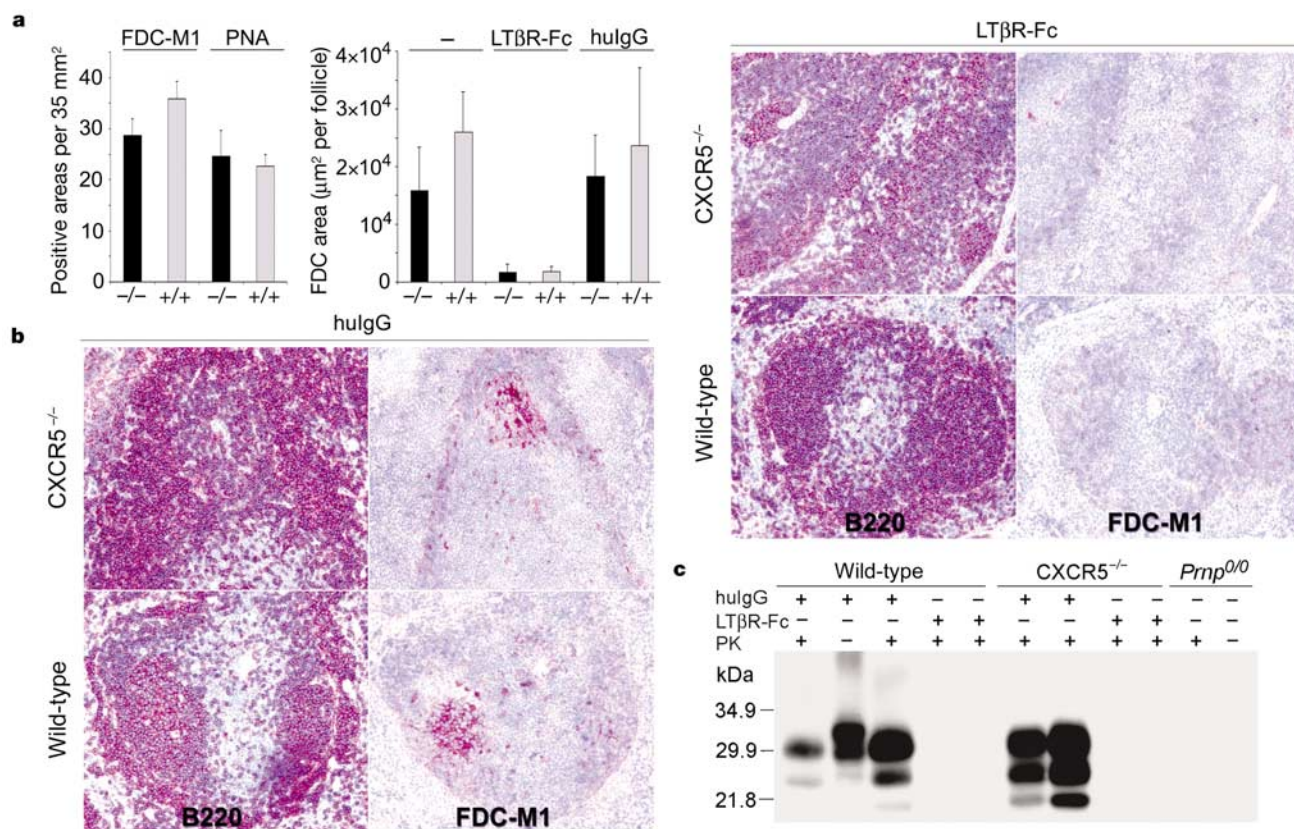


Figure 3 Splenic prion replication in CXCR5^{-/-} mice in the absence of LTβR signalling. **a**, CXCR5^{-/-} (-/-) and WT (+/+) mice were treated repeatedly with pooled human IgG (hulG) or soluble LTβR (LTβR-Fc). Quantitative analysis of germinal-centre induction as measured by PNA stain and analysis of FDC-M1 immunoreactive networks in prion-infected mice (35 d.p.i.) (left panel). PNA⁺ and FDC-M1⁺ positive areas were counted in 35 mm² of independent spleen sections. Data represent mean ± s.d. of three mice for each group. Right panel shows FDC-M1 immunoreactive areas of untreated, LTβR-Fc and human-IgG-treated mice. Size was assessed by morphometric measurement of FDC-M1⁺ areas (μm² follicle). Data represent means ± s.d. of >2 spleens in each

experimental group. **b**, Spleens of CXCR5^{-/-} (top row) and WT (bottom row) mice treated repeatedly with pooled human IgG or soluble LTβR (LTβR-Fc). LTβR-Fc treatment abolished FDC-M1 immunoreactivity in both CXCR5^{-/-} and WT spleens. **c**, Western blot analysis of human-IgG- or LTβR-Fc-treated WT or CXCR5^{-/-} spleens. Mice were inoculated i.p. with 5 log LD₅₀ scrapie prions, and spleens were analysed 38 days after infection. Tissue extracts were electrophoresed natively (-) or after digestion with proteinase K (PK) (+). PK-resistant prion protein (PrP^{Sc}) was undetectable in LTβR-Fc treated mice of either genotype.

alive at the time of writing, Fig. 4a). The combined lines of evidence described above exclude a number of possible alternative scenarios, and strongly suggest that acceleration of pathogenesis in CXCR5^{-/-} mice is due to mislocalized FDCs.

CXCR5^{-/-} mice lack inguinal lymph nodes and most Peyer's patches^{13,23}, and may suffer from stromal defects affecting prion pathogenesis. This was investigated in reciprocal bone marrow chimaeras: CXCR5^{-/-} → CXCR5^{-/-}; CXCR5^{-/-} → WT; WT → WT; and WT → CXCR5^{-/-}. Six to eight weeks after bone marrow grafting, peripheral blood was taken and assessed for successful reconstitution by fluorescence-activated cell sorting (FACS) analysis for the presence or absence of CXCR5 (Fig. 4b). Wild-type mice had ≥72%, and CXCR5^{-/-} mice had ≤10% B220⁺CXCR5⁺ lymphocytes. For WT → CXCR5^{-/-} reconstitutions, only chimaeric mice with ≥68% of B220⁺CXCR5⁺ cells were used for subsequent experiments. In CXCR5^{-/-} → WT reconstitutions, only chimaeric mice harboring ≤12% of B220⁺CXCR5⁺ cells were used.

Wild-type B cells, as expected, migrated and settled properly in primary follicles antipodally to central arterioles of white pulp follicles of CXCR5^{-/-} recipients, whereas perivascular FDC-M1⁺ and CD35⁺ networks were abrogated in these mice. Therefore, recipient follicles had no stromal defects (Fig. 4c, top row). Occasional small B-cell clusters remained around a few arterioles,

perhaps indicative of residual radioresistant cells. In CXCR5^{-/-} → WT chimaeric mice, B cells formed *de novo* periarteriolar FDC-M1⁺ and CD35⁺ cuffs (Fig. 4c, bottom row) although pre-existing follicles did not completely convert to the CXCR5^{-/-} phenotype. These results confirm that B cells, even when homing to atypical structures, maintain their ability to define the localization of primary follicles and FDCs.

We then challenged chimaeras i.p. with 3 log LD₅₀ scrapie prions. At 35 d.p.i., chimaeric spleens of all groups (two spleens per group) showed very similar infectivity content (3.5–4.5 log LD₅₀), except for one single CXCR5^{-/-} → WT outlier with 2.4 log LD₅₀ (data not shown), indicating that bone marrow reconstitutions do not impair splenic prion replication.

At least four mice per group were allowed to progress to terminal disease (Fig. 4d). WT → CXCR5^{-/-} chimaeras developed scrapie at 241 ± 10 d.p.i., similarly to WT → WT chimaeras (237 ± 9 d.p.i.). Instead, CXCR5^{-/-} → WT (208 ± 9 d.p.i.; *P* < 0.01 compared with WT → WT chimaeras) and CXCR5^{-/-} → CXCR5^{-/-} (211 ± 12 d.p.i.; *P* < 0.01 compared with WT → CXCR5^{-/-}) chimaeras developed scrapie significantly earlier. This confirms that alteration of B-cell homing behaviour controls the speed of prion neuroinvasion by altering the relationship between FDCs and sympathetic nerves.

The above results implicate topographical relationships between

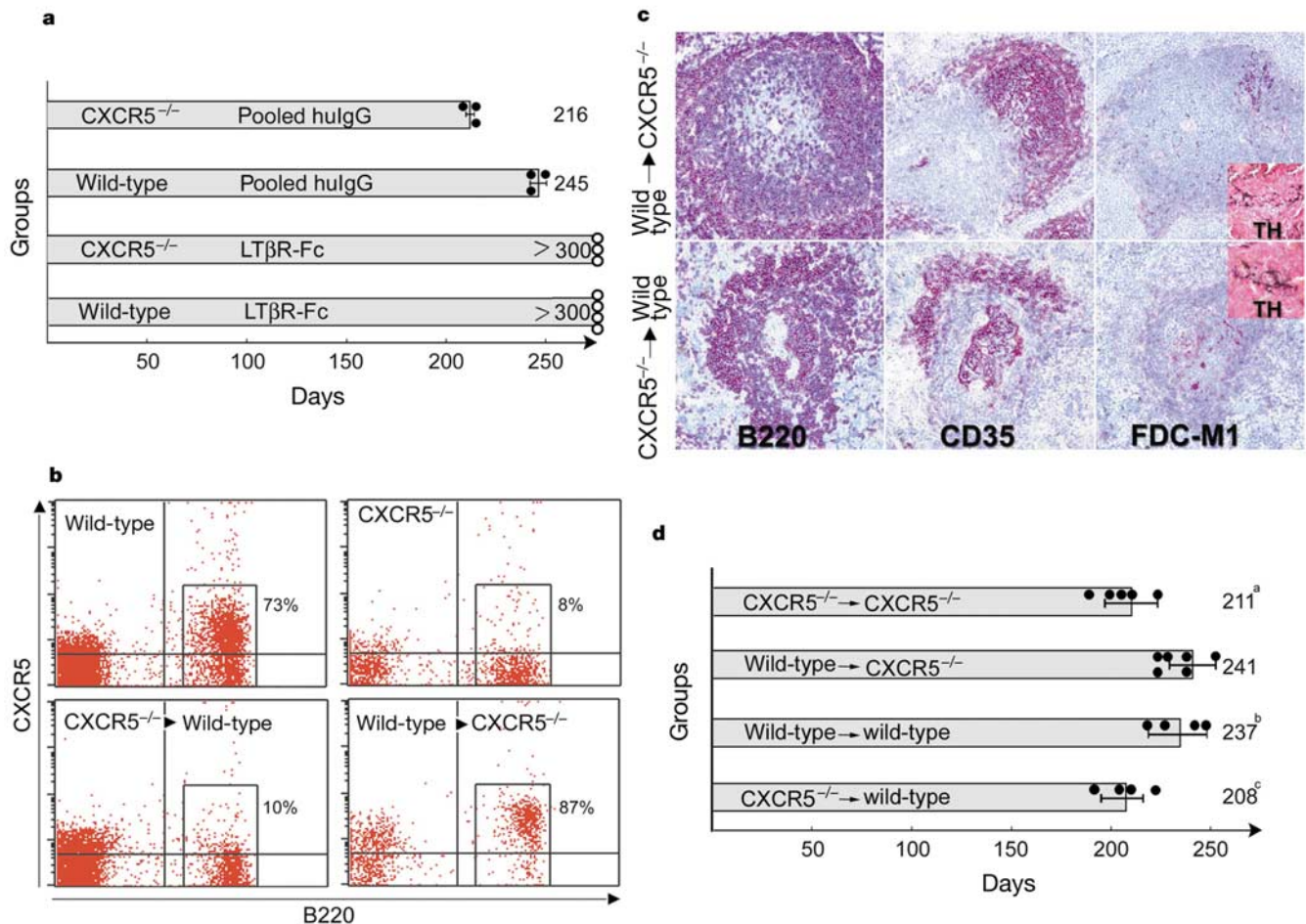


Figure 4 FDC localization and prion replication in LTβR-Fc treated mice and bone-marrow chimaeras. **a**, Effect of LTβR-Fc treatment on prion incubation times in CXCR5^{-/-} mice. Black circles indicate individual CXCR5^{-/-} or wild-type mice challenged with 3 log LD₅₀ succumbing scrapie. Hollow symbols indicate that mice were still alive at the time of writing this manuscript. Bar lengths and numbers indicate average ± s.e. of incubation time. **b**, FACS analysis of peripheral blood cells 6–8 weeks after bone-marrow reconstitution in chimaeric mice or in WT and CXCR5^{-/-} mice. B220⁺CXCR5⁺ cells appear in the top right quadrant of each plot. Lymphocyte populations were gated according to size and granularity in forward and side scattergrams (not shown). **c**, Histology of spleens of WT → CXCR5^{-/-} and CXCR5^{-/-} → WT

chimaeric mice. WT bone marrow transfer to CXCR5^{-/-} hosts (top row) suppressed perivascular FDCs, while CXCR5^{-/-} bone marrow transfer to WT hosts (bottom row) restored them (100 ×). Insets, TH immunostains of the respective chimaeras (200 ×). **d**, Scrapie kinetics in chimaeric mice. Black circles indicate individual chimaeras challenged with 3 log LD₅₀ succumbing scrapie. Bar lengths and numbers indicate average ± s.e. of incubation time. A few mice (see superscripts a, b and c) succumbed to secondary infections after bone marrow grafting at these time points. a: 44, 44, 58 days; b: 72, 131 days; c: 33 days. None of these mice showed any clinical or histological signs of scrapie.

FDCs and sympathetic nerve endings as determinants of prion neuroinvasion. Re-engineered follicles with a shortened distance between these elements selectively increase the velocity of neuroinvasion upon peripheral challenge. How might prions transit from FDCs to sympathetic nerve fibres? Prion-infected FDCs may degenerate and liberate prions, which then diffuse passively and occasionally hit peripheral nerve endings, thereby triggering uptake and neuroinvasion. Alternatively, mobile cells may traffic prions between FDCs and peripheral nerves. In the first case, efficiency of neuroinvasion may inversely correlate with the distance between FDCs and nerves. By contrast, active prion transport may result in complex patterns and should be modifiable by manipulation of the cells involved.

Human CJD can be diagnosed before death on lymphoid tissues²⁴, and patients may display lymphoreticular PrP^{Sc} long before neuroinvasion²⁵. The 'neuroimmune synapse' in prion pathogenesis is important, since efficiency of neuroimmune transfer may determine whether a given individual will develop disease upon prion infection. The growing family of immunoreagents that affect

homeostasis of stromal FDCs may add to the rational strategies²⁶ for antiprion intervention. Conversely, endogenous or exogenous modifiers of FDC localization may act as modifiers of susceptibility to prion diseases. □

Methods

Prion inoculations

CXCR5^{-/-} and K14NGF mice have been described previously^{13,16}. The genetic strain background of all mice was 129 Sv. Animals were maintained under specific pathogen-free conditions and infected i.p. with 100 μl of brain homogenate diluted in PBS with 5% BSA and containing 3–6 log LD₅₀ units of the Rocky Mountain laboratory (RML) scrapie strain (passage 5, hence called RML5). For control, *tga20* mice were infected i.p. with 100 μl of brain homogenate diluted in PBS with 5% BSA containing 3 or 6 log LD₅₀ units of RML5. The titre of this standard RML5 inoculum was 8.9 log LD₅₀ g⁻¹ of brain tissue¹⁸. For i.c. inoculations, 30 μl of inoculum with 3 × 10⁵ LD₅₀ or 300 LD₅₀ were administered. Scrapie was diagnosed according to clinical criteria (ataxia, kyphosis, priapism and hind-leg paresis). Mice were killed on the day of onset of terminal clinical signs of scrapie.

Infectivity bioassays

Assays were performed on 1% (w/v) spleen, mesenteric or superficial cervical lymph nodes or thoracic spinal cord (segments 7–9) homogenates. Tissues were homogenized in 0.32 M

sucrose diluted in PBS containing 5% BSA. When the solution appeared homogeneous, it was spun for 5 min at 500g. Supernatants (30 µl) were inoculated i.c. into groups of four *tga20* mice¹⁹. Infectivity titres were then determined using the relationship $y = 11.45 - 0.088x$ (y , log LD₅₀ per millilitre of homogenate; x , incubation time in days to terminal disease), which was derived by linear regression using Prusiner's method¹⁷.

Western blot analysis

Tissue homogenates were adjusted to 5 mg ml⁻¹ protein and run natively or after treatment with proteinase K (50 µg ml⁻¹, 30 min, 37 °C). 50 µg of total protein were electrophoresed through 12% SDS-PAGE and blotted. Membranes were incubated with monoclonal antibody ICSM18 to PrP (1:20,000)²⁷ or TH-2 to tyrosine hydroxylase (1:5,000; Sigma), and visualized by enhanced chemiluminescence (ECL; Amersham).

Histology and immunohistochemistry

Paraffin (2 µm) and frozen sections from brain (10 µm) or spleen (5 µm) were stained with haematoxylin/eosin. Immunostaining for glial fibrillary acidic protein (GFAP) was performed with a rabbit antiserum (1:300 dilution; DAKO) and detected with biotinylated swine anti-rabbit serum (1:250 dilution; DAKO) and diaminobenzidine (Sigma). Antibodies FDC-M1 (clone 4C11; 1:50; Becton Dickinson), CD35 (8C12; Pharmingen) and anti-CD45RO/B220 (RA3-6B2; Pharmingen) were used as described²⁸. For the assessment of splenic innervation, floating sections were blocked in 4% goat serum, incubated overnight with a polyclonal anti-TH antibody (1:400; Fluka), washed, and incubated with a biotinylated secondary antibody (1:100 goat anti rabbit; Vector). Visualization was accomplished with nickel-enhanced diaminobenzidine. Morphometric analysis was performed using Analysis Pro 3.1 software (Soft Imaging System; Münster). FDCs were quantified by measuring the FDC-M1⁺ surface in ≥48 WT and CXCR5^{-/-} follicles. For LTβR-Fc and human-IgG-treated mice, ≥12 follicles were examined.

Treatment with LTβR fusion protein

Mice were treated i.p. with 250 µg LTβR-Ig fusion protein²⁹ one week before prion inoculation. Injection was repeated 14 and 28 days later. For control, Sv129 mice were subjected to the same regimen with 250 µg of a human IgG polyclonal preparation (Biogen) instead of LTβR-Ig. Two mice from each group were analysed for prion infectivity and PrP^{Sc} content 38 days after inoculation.

Bone marrow chimaeric mice

5 × 10⁶ bone marrow cells were isolated from tibiae and femurs, and injected into tail veins of 8–10-week-old recipients conditioned by whole-body irradiation (1,100 rad) 24 h earlier. 6–8 weeks after grafting, reconstitution was assessed by FACS analysis of peripheral blood taken from the retro-orbital plexus of ether-anaesthetized mice. Blood samples were prepared at 4 °C in buffer solution (PBS containing 2% FCS and 0.2% NaN₃) and stained with fluorescein isothiocyanate (FITC)-labelled CD45R/B220 (Pharmingen) and phycoerythrin (PE)-labelled anti-CXCR5 (clone 2G8; ref. 13). After lysis of erythrocytes with FACS lysis solution (Becton Dickinson) and washing, cell suspensions were analysed with a FACScalibur (Becton Dickinson). Viable cells were gated by forward and side light scatter. Data were acquired with CellQuest software (Becton Dickinson). The following markers were used: anti-B220-PerCP, anti-CD21-FITC, anti-CD23-bio, anti-CD23-PE, anti-CD18-PE, anti-CD29-biotin, anti-CD49d-biotin, anti-CD5-bio, anti-CD11b (Mac-1)-FITC, anti-CD11a-biotin and streptavidin-APC (all from BD-Pharmingen). For characterization of marginal zone-B (MZB) and follicular B (FB) cells, a combination of anti-B220/CD21/CD23 was used. For detection of integrin expression on MZB cells, anti-CD49d (anti-α4), anti-CD11a (anti-αLβ2), anti-CD18 (anti-β2) and anti-CD29 (anti-β1) antibodies were combined with the anti-B220/CD21/CD23 stain. For identification of peritoneal B-1 and B-2 cells, a combination of anti-B220/CD5/CD11b antibodies was used.

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Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation

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Stem cells persist throughout life by self-renewing in numerous tissues including the central¹ and peripheral² nervous systems. This raises the issue of whether there is a conserved mechanism to effect self-renewing divisions. Deficiency in the polycomb family transcriptional repressor *Bmi-1* leads to progressive post-natal growth retardation and neurological defects³. Here we show that *Bmi-1* is required for the self-renewal of stem cells in the peripheral and central nervous systems but not for their survival or differentiation. The reduced self-renewal of *Bmi-1*-deficient neural stem cells leads to their postnatal depletion. In the absence of *Bmi-1*, the cyclin-dependent kinase inhibitor gene *p16^{Ink4a}* is

upregulated in neural stem cells, reducing the rate of proliferation. *p16^{Ink4a}* deficiency partially reverses the self-renewal defect in *Bmi-1*^{-/-} neural stem cells. This conserved requirement for *Bmi-1* to promote self-renewal and to repress *p16^{Ink4a}* expression suggests that a common mechanism regulates the self-renewal and postnatal persistence of diverse types of stem cell. Restricted neural progenitors from the gut and forebrain proliferate normally in the absence of *Bmi-1*. Thus, *Bmi-1* dependence distinguishes stem cell self-renewal from restricted progenitor proliferation in these tissues.

Bmi-1 is an oncogene that causes neoplastic proliferation when overexpressed in lymphocytes^{4,5}. Deletion of *Bmi-1* leads to defects in axial skeleton patterning and haematopoiesis, and to ataxia and seizures³. *Bmi-1*-deficient mice can survive into adulthood but show progressive postnatal growth retardation (ref. 3 and Supplementary Fig. 1), raising the possibility that *Bmi-1* regulates stem cell function. Two studies have shown that *Bmi-1* is required for the postnatal maintenance of haematopoietic stem cells^{6,7}. In those studies, however, it was not technically possible in the haematopoietic system to discern directly whether the failure of stem cell maintenance was caused by a defect in self-renewal or survival.

To determine whether *Bmi-1* regulates the self-renewal of mouse neural stem cells, we examined the effect of *Bmi-1* deficiency on the

self-renewal of central nervous system (CNS) stem cells from the telencephalon at embryonic day 14.5 (E14.5)⁸ and neural crest stem cells (NCSCs) from the gut at E14.5 (enteric nervous system)⁹. CNS stem cells form CNS neurospheres, whereas gut NCSCs form PNS neurospheres in non-adherent cultures (Fig. 1 and Supplementary Fig. 3). CNS and PNS neurospheres and purified uncultured p75⁺α₄⁺ rat gut NCSCs⁹ expressed *Bmi-1* (Supplementary Fig. 2 and data not shown). *Bmi-1*^{-/-} telencephalon cells formed multipotent neurospheres, but at a significantly lower frequency ($P < 0.05$) than did wild-type telencephalon cells (Fig. 1). This suggests that there are significantly fewer stem cells in the *Bmi-1*^{-/-} telencephalon. The *Bmi-1*^{-/-} neurospheres were also roughly 14-fold smaller in volume ($P < 0.01$) and gave rise to 33-fold fewer ($P < 0.01$) secondary neurospheres on subcloning, indicating a defect in self-renewal. In the E14.5 PNS, the frequency of cells capable of forming neurospheres did not differ between wild-type and *Bmi-1*^{-/-} guts, but the *Bmi-1*^{-/-} neurospheres were significantly smaller and gave rise to 60-fold fewer secondary multipotent neurospheres ($P < 0.01$) on subcloning (Fig. 1). Thus, *Bmi-1* deficiency reduced the self-renewal of both CNS stem cells and NCSCs in culture.

If neural stem cells require *Bmi-1* for normal self-renewal *in vivo*, the stem cells in *Bmi-1*-deficient mice should become depleted postnatally. In the absence of *Bmi-1*, the frequency of lateral

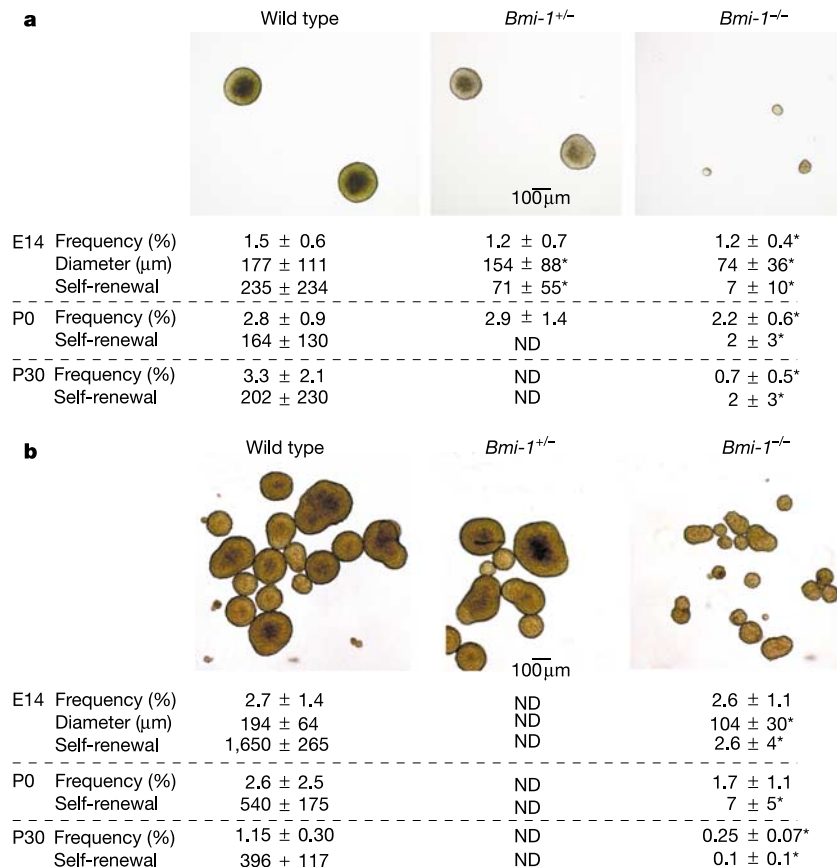


Figure 1 CNS stem cells and gut neural crest stem cells (NCSCs) require *Bmi-1* to self-renew normally. Images show typical neurospheres that formed after 10 d in non-adherent cultures from E14.5 CNS neural stem cells (**a**) or PNS NCSCs (**b**). **a**, The frequency of *Bmi-1*^{-/-} E14 telencephalon cells, P0 SVZ cells or P30 SVZ cells that formed multipotent CNS neurospheres was significantly reduced relative to wild-type cells ($*P < 0.05$). The diameter and self-renewal of the *Bmi-1*^{-/-} neurospheres were also significantly reduced. Self-renewal capacity is expressed as the number of secondary neurospheres generated per primary neurosphere on subcloning. **b**, Similar results were

obtained for multipotent PNS neurospheres generated by E14.5, P0 or P30 wild-type and *Bmi-1*^{-/-} gut cells, except that the frequency of neurosphere-forming cells was not significantly reduced until P30. Values are the mean ± s.d. for 3–6 independent experiments. *Bmi-1* deficiency also consistently reduced self-renewal when the data were normalized to control for differences in the size of neurospheres: $3.5 \pm 0.8\%$ of dissociated wild-type P0 PNS neurosphere cells formed secondary neurospheres on subcloning, as compared with $0.3 \pm 0.1\%$ of *Bmi-1*^{-/-} cells ($P < 0.001$). ND, not determined.

ventricle subventricular zone (SVZ) cells and gut cells that were capable of forming multipotent neurospheres in culture was modestly reduced at postnatal day 0 (P0) and strongly reduced at P30 (Fig. 1). Similar trends were observed in terms of the ability of the cells to form multilineage adherent colonies in culture (data not shown). The extent to which *Bmi-1* deficiency reduced the self-renewal potential of stem cells increased over time (Fig. 1). Thus, *Bmi-1* is required for the self-renewal of stem cells in diverse tissues, and *Bmi-1*^{-/-} stem cells rarely persist into adulthood.

Consistent with the reduction in neurosphere size (Fig. 1), *Bmi-1*^{-/-} neural stem cell colonies contained one-third to one-tenth as many cells as did wild-type colonies (Fig. 2a). We were unable to detect any increase in cell death in *Bmi-1*^{-/-} stem cell colonies or in the SVZ of the lateral ventricle *in vivo* (Fig. 2b–d). By contrast, the proliferation of cells in stem cell colonies was significantly reduced (Fig. 2e, f). Thus, the reduced self-renewal of *Bmi-1*^{-/-} neural stem cells was caused at least partly by reduced proliferation.

To assess the effect of *Bmi-1* on proliferation *in vivo*, we examined the rate of 5-bromodeoxyuridine (BrdU) incorporation into P0 and P30 SVZ cells. As compared with wild-type SVZ cells, *Bmi-1*^{-/-} SVZ cells showed a significantly lower rate of BrdU incorporation at both ages (17.8 ± 0.5% (*Bmi-1*^{-/-}) versus 19.9 ± 2.0% (wild type) of SVZ cells were BrdU-positive at P0; *P* < 0.05), although the effect was greater at P30 (13.1 ± 2.7% versus 20.1 ± 0.5%; *P* < 0.01).

Bmi-1 deficiency is associated with increased expression of the

p16^{Ink4a} and *p19*^{Arf} genes encoding cyclin-dependent kinase inhibitors, and deletion of these genes partially rescues the growth of *Bmi-1*^{-/-} mice^{10–12}. Analysis by quantitative real-time polymerase chain reaction (qRT-PCR) showed that *p16*^{Ink4a} expression increased 5–21-fold, whereas *p19*^{Arf} expression increased 1.4–3-fold, in *Bmi-1*^{-/-} CNS and PNS neurospheres of all ages (Supplementary Table 1 and Supplementary Figs 4f and 5f). To test whether *p16*^{Ink4a} regulates neural stem cell self-renewal, we examined neurospheres cultured from *p16*^{-/-} adult mice (ref. 13 and Fig. 3). *p16*^{-/-} neurospheres from both the CNS and PNS were larger and self-renewed to a significantly greater extent than did wild-type neurospheres (Fig. 3a, b). Adherent CNS and PNS *p16*^{-/-} stem cell colonies contained more cells and incorporated BrdU at a higher rate (Fig. 3c–f). These data indicate that *p16*^{Ink4a} inhibits neural stem cell self-renewal in culture, although the magnitude of this effect may depend on culture conditions, because *p16*^{Ink4a} can be induced in response to stress¹⁴.

To address directly whether *Bmi-1* promotes neural stem cell self-renewal by suppressing the expression of *p16*^{Ink4a}, we generated *Bmi-1*^{-/-}*p16*^{-/-} mice. *p16*^{Ink4a} deficiency significantly increased the self-renewal of *Bmi-1*^{-/-} CNS stem cells and NCSCs in culture (*P* < 0.01), but did not fully restore self-renewal to wild-type levels (Fig. 3g, h). This indicates that there are additional pathways downstream of *Bmi-1* that regulate self-renewal, perhaps including *p19*^{Arf}. The increased self-renewal of *Bmi-1*^{-/-}*p16*^{-/-} CNS stem cells and NCSCs was associated with a significant increase in the rate of proliferation in stem cell colonies (23.5 ± 11.1% BrdU-

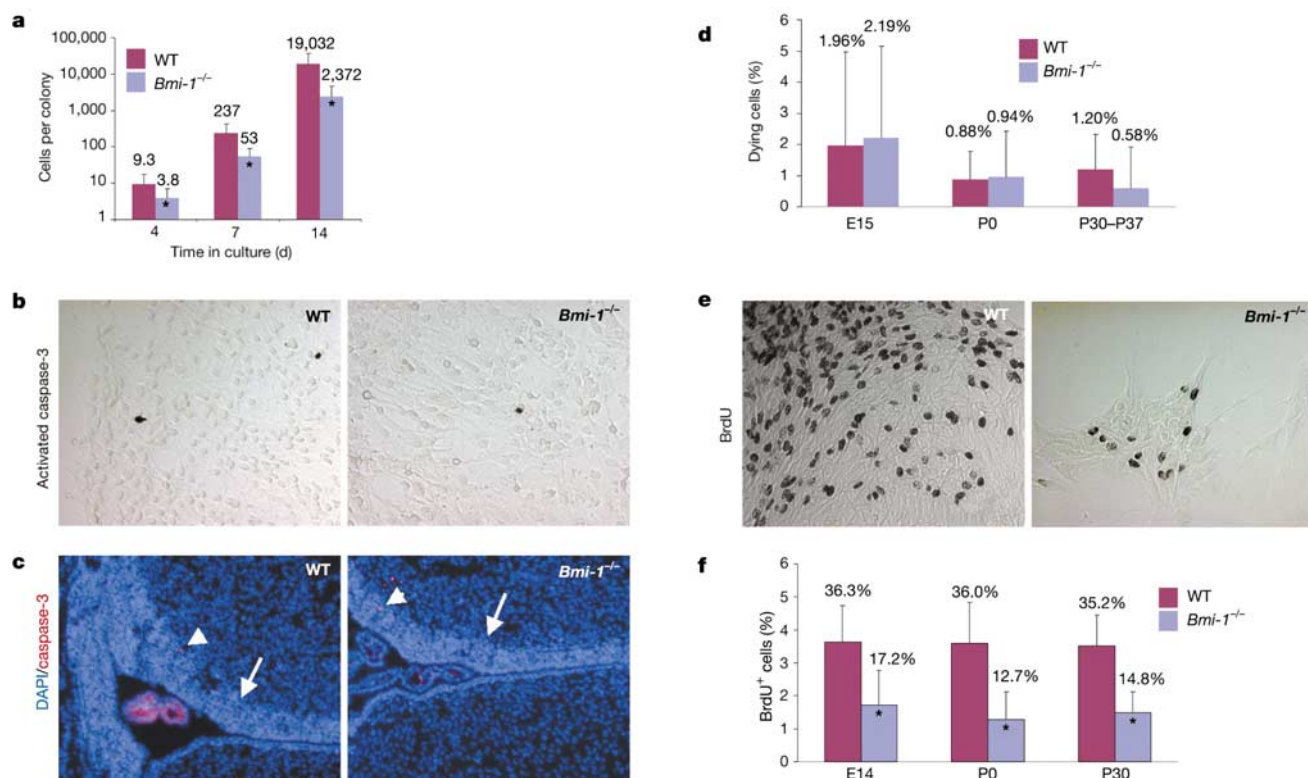


Figure 2 *Bmi-1* deficiency reduces proliferation but does not increase cell death in CNS stem cell colonies. P0 SVZ cells were dissociated and plated in adherent cultures, and the number of cells per colony was counted after 4, 7 and 14 d (**a**). All colonies were counted after 4 d, but only colonies that contained neurons and glia were counted after 7 and 14 d. At all time points, wild-type (WT) colonies contained significantly more cells than the *Bmi-1*^{-/-} colonies (**P* < 0.01). Similar results were obtained at E14 and P30 (not shown). Only a few cells in adherent CNS stem cell colonies (**b**) or in the P0 SVZ (**c**) stained with antibodies against activated caspase-3 (a marker of cell death). Arrows and arrowheads indicate the SVZ and caspase-3-positive cells, respectively. Note that the

choroid plexus in the lateral ventricle is highly autofluorescent (**c**). No statistically significant difference in the frequency of dying cells was observed between wild-type and *Bmi-1*^{-/-} CNS stem cell colonies on the basis of the frequency of condensed, fragmented nuclei identified by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (**d**), or activated caspase-3 (not shown). *Bmi-1* deficiency was associated with a significant decrease in the rate of BrdU incorporation into CNS stem cell colonies (**e**, **f**; **P* < 0.01), indicating reduced proliferation. A similar reduction in proliferation was observed in *Bmi-1*^{-/-} NCSC colonies in culture (not shown).

positive cells versus $8.9 \pm 11.6\%$ in $Bmi-1^{-/-}p16^{+/+}$ CNS stem-cell colonies, $P < 0.01$). Therefore, the increase in $p16^{Ink4a}$ expression in the absence of $Bmi-1$ contributed to the reduced self-renewal of $Bmi-1^{-/-}$ neural stem cells in culture.

Because $p16^{Ink4a}$ and $p19^{Arf}$ expression can increase in cultured cells¹⁵, we examined whether these genes were also upregulated in uncultured progenitors (Supplementary Table 1). Expression of $p16^{Ink4a}$ RNA and protein was increased in the P0 and P30 SVZ of $Bmi-1^{-/-}$ mice but was not detected in the E15 telencephalon (Supplementary Table 1 and Supplementary Fig. 4e). $p16^{Ink4a}$ was also upregulated in uncultured $Bmi-1^{-/-}$ P30 SVZ FSC^{hi}SSEA-1^{hi}CD24^{-/lo} cells, which are enriched for CNS stem cells^{16,17}. Expression of $p19^{Arf}$ followed similar trends but was upregulated to a lesser extent than $p16^{Ink4a}$ (Supplementary Table 1). In the PNS, $p16^{Ink4a}$ and $p19^{Arf}$ followed trends similar to those observed in the CNS. Expression of $p16^{Ink4a}$ was also increased in P0 and P30 uncultured $Bmi-1^{-/-}p75^{+}$ gut cells, which are enriched for

NCSCs². Consistent with the increased expression of $p16^{Ink4a}$ in the nervous system of $Bmi-1$ -deficient mice *in vivo*, $p16$ deficiency partially rescued at least some aspects of nervous system development in $Bmi-1^{-/-}$ mice. For example, the number of neurons per section through the P30 distal small intestine was 92 ± 30 (mean $\pm$ s.d.) for wild-type, 47 ± 19 for $Bmi-1^{-/-}$ and 78 ± 18 for $Bmi-1^{-/-}p16^{-/-}$ mice (all differences $P < 0.05$).

These data suggest that $Bmi-1$ is required in the nervous system *in vivo* to repress $p16^{Ink4a}$ and $p19^{Arf}$ postnatally but not during fetal development. The lack of $p16^{Ink4a}$ or $p19^{Arf}$ expression in the $Bmi-1^{-/-}$ telencephalon may be a reason why forebrain development seems relatively normal in $Bmi-1^{-/-}$ newborns despite the poor proliferation of E14.5 $Bmi-1^{-/-}$ neurospheres, which do express $p16^{Ink4a}$ in culture. Nonetheless, $p16^{Ink4a}$ expression was increased *in vivo* in the $Bmi-1^{-/-}$ SVZ at P0 and P30, consistent with the proliferation defect observed in the postnatal SVZ and with the postnatal depletion of stem cells.

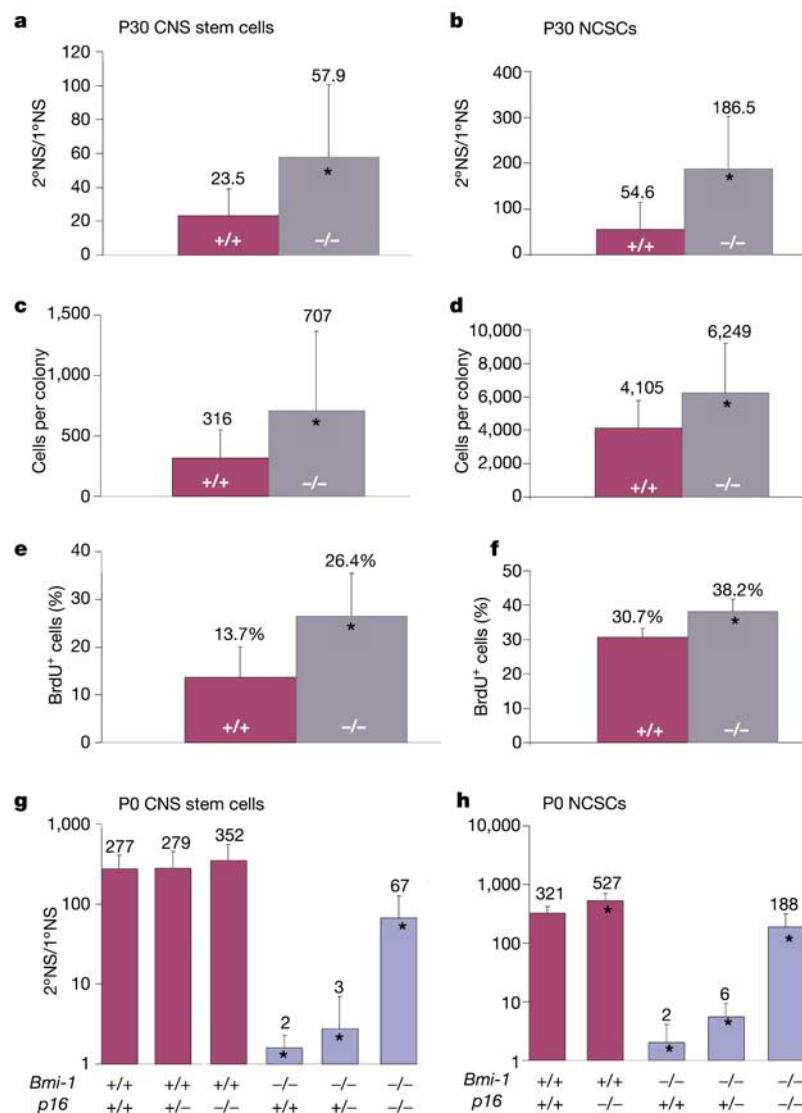


Figure 3 $p16^{Ink4a}$ negatively regulates the self-renewal of CNS stem cells and gut NCSCs in culture. SVZ and gut cells from adult $p16^{-/-}$ and wild-type mice were dissociated and cultured to generate neurospheres (a, b) or adherent stem cell colonies (c–f). After 7 d (a, c, e) or 10 d (b, d, f) in culture, self-renewal (a, b), the total number of cells per colony (c, d), and the percentage of BrdU-positive cells per colony (e, f) were assayed. In each case, the $p16^{-/-}$ adult neural stem cell colonies self-renewed more, proliferated more and contained more cells ($*P < 0.05$). No differences were observed between $p16^{-/-}$

and wild-type colonies in the frequency of apoptotic cells ($0.59 \pm 0.61\%$ versus $0.35 \pm 0.48\%$ respectively). $Bmi-1^{+/+}p16^{+/-}$ mice were also bred to generate P0 pups, from which SVZ CNS stem cells (g) and gut NCSCs (h) were cultured. For both types of cell, $p16^{Ink4a}$ deficiency significantly increased the self-renewal of $Bmi-1^{-/-}$ stem cells ($*P < 0.01$), although it did not fully restore self-renewal to wild-type levels ($*P < 0.01$ relative to wild type). Note that $p16^{Ink4a}$ deficiency in $Bmi-1^{+/+}$ stem cells significantly increased the self-renewal of P0 NCSCs (h) but not P0 CNS stem cells (g).

The proliferation of restricted neural progenitors might be regulated differently from that of stem cells because restricted progenitors are not multipotent and divide a limited number of times before becoming postmitotic. To test whether these cells are dependent on *Bmi-1*, we cultured E14 telencephalon cells, or P0 or P30 SVZ cells, at clonal density under adherent conditions. In contrast to the significantly reduced frequency of stem cells in *Bmi-1*^{-/-} samples (Fig. 1), the frequency of cells that formed neuron-only or glia-only colonies did not significantly differ between wild-type and *Bmi-1*^{-/-} samples (Fig. 4a). There were also no significant differences in the number of cells per neuron-only or glia-only colony from the CNS or PNS (Fig. 4b–e). Thus, *Bmi-1*^{-/-} restricted neural progenitors were present in relatively normal numbers and proliferated normally in culture.

To test whether lineage commitment caused the proliferation of neural progenitors to become *Bmi-1* independent, we cultured E14 gut p75⁺ neural crest cells at clonal density for 4 d with or without bone morphogenetic protein 4 (BMP4), which instructs gut NCSCs to undergo neurogenesis^{9,18,19}. In the absence of BMP4 no neuron-only colonies were observed in these cultures, but in the presence of BMP4 significant numbers of neuron-only colonies emerged. The number of cells per neuron-only colony did not differ between wild-type and *Bmi-1*^{-/-} cultures (Fig. 4e).

Neuregulin (Nrg) promotes the proliferation of glial progenitors

in addition to instructing postnatal gut NCSCs to acquire a glial fate^{2,19,20}. To test whether *Bmi-1*^{-/-} glial progenitors proliferated normally when stimulated by Nrg, we cultured dissociated P0 gut cells in adherent cultures at clonal density with or without Nrg for 6 d. In the absence of Nrg, *Bmi-1*^{-/-} colonies incorporated BrdU (pulsed on day 6) at only a third of the rate of wild-type colonies; however, in the presence of Nrg, *Bmi-1*^{-/-} colonies incorporated BrdU at almost the same rate as wild-type colonies (Fig. 4f). These data indicate that lineage determination factors change the cell-cycle regulation of stem cells in addition to restricting their developmental potential, causing the proliferation of neural progenitors to become *Bmi-1* independent.

Bmi-1 was expressed in restricted neural progenitors and *p16*^{Ink4a} RNA and protein expression remained increased in *Bmi-1*^{-/-} restricted neural progenitor colonies (Supplementary Fig. 5f); however, *p16*^{Ink4a} deficiency did not affect the frequency or proliferation of restricted neural progenitors (Supplementary Fig. 5). Thus, lineage restriction was associated with a change in cell-cycle regulation that caused restricted neural progenitors to proliferate in culture in a way that was not affected by *Bmi-1* or *p16*^{Ink4a} deficiency.

The reduced stem cell frequency in the *Bmi-1*^{-/-} telencephalon (Fig. 1) indicates that other pathways downstream of *Bmi-1* may also regulate self-renewal, because neither *p16*^{Ink4a} nor *p19*^{Arf} was

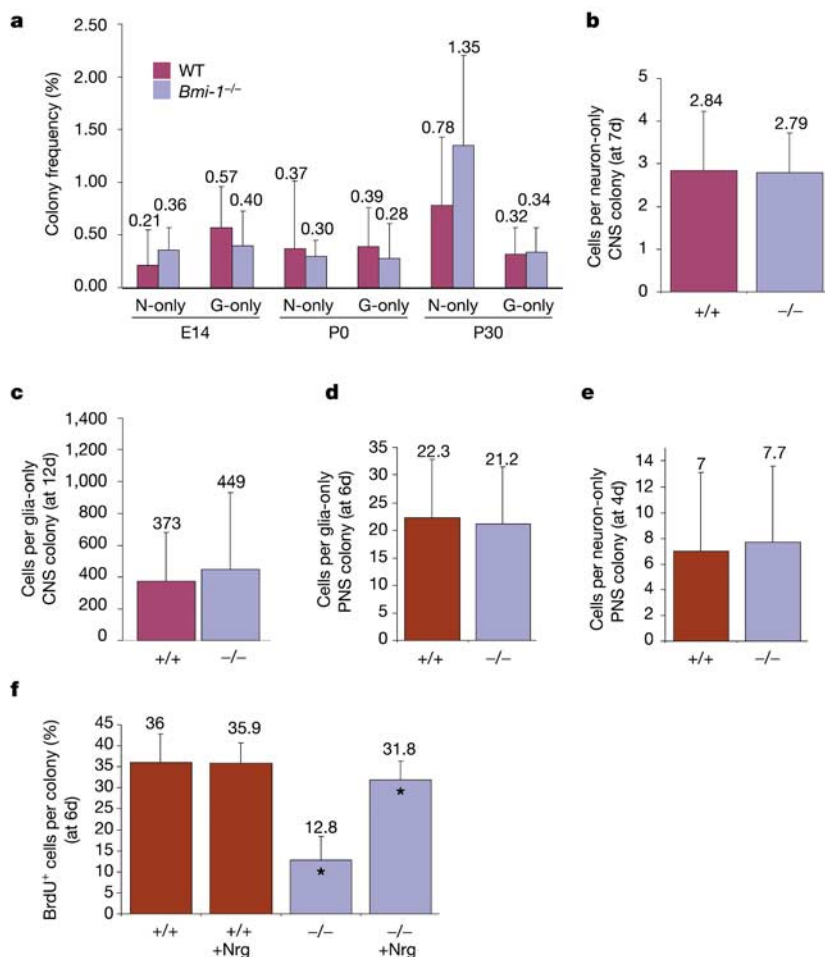


Figure 4 Restricted neural progenitors from the CNS and PNS proliferate normally in the absence of *Bmi-1*. E14 telencephalon cells or P0 or P30 SVZ cells were dissociated and cultured at clonal density under adherent conditions. **a–c**, The frequency of CNS cells that formed neuron-only or glia-only colonies after 12–14 d in culture did not significantly differ between wild-type (WT) and *Bmi-1*^{-/-} mice (**a**), nor did the number of cells per neuron-only colony (**b**) or glia-only colony (**c**). **d**, The number of cells per glia-only colony

formed by dissociated P0 gut cells did not differ between wild-type and *Bmi-1*^{-/-} mice. **e**, In BMP4-treated cultures of E14.5 gut p75⁺ cells, no difference was observed in the number of cells per neuron-only colony. **f**, *Bmi-1*^{-/-} neural progenitor colonies in the absence of Nrg proliferated at a significantly lower rate than did wild-type colonies (**P* < 0.01), but *Bmi-1*^{-/-} colonies in the presence of Nrg proliferated similarly to wild-type colonies (although the difference was still statistically significant, **P* < 0.05).

expressed in the telencephalon (Supplementary Table 1). To identify additional pathways that are regulated directly or indirectly by *Bmi-1*, we compared the gene expression profiles of wild-type and *Bmi-1*^{-/-} neurospheres that had been cultured from the P0 SVZ or from the P0 gut. Because *Bmi-1* is a repressor^{21,22}, and the function of *Bmi-1* seems to be conserved between CNS and PNS stem cells, we examined whether any genes were significantly upregulated (fold change >2; *P* < 0.05) in *Bmi-1*^{-/-} neurospheres from both the CNS and PNS. We found 11 genes that met these criteria and that were confirmed to be expressed differentially in independent samples by qRT-PCR (out of 36,701 probe sets examined; Supplementary Table 2). In addition to *p16*^{Ink4a} and *p19*^{Arf}, these included genes that were previously identified as being regulated by *Bmi-1*, such as Hox genes (*hoxD8*, *hoxD9* and *hoxC9*)^{7,23,24}, and several genes that have not been previously identified as being regulated by *Bmi-1*, such as the cyclin-dependent kinase inhibitor gene *p21*, which regulates haematopoietic stem cell self-renewal²⁵, and *Gas6*, which encodes a ligand for the Axl receptor tyrosine kinase²⁶. More work will be required to determine whether any of these genes regulate neural stem cell self-renewal.

Although *Bmi-1* was found to be consistently required for the self-renewal of haematopoietic stem cells^{6,7}, CNS stem cells and NCSCs, it was not required for the proliferation of restricted neuronal and glial progenitors from the forebrain or from the enteric nervous system (Fig. 4). It is not the case that all restricted progenitors proliferate normally in the absence of *Bmi-1*, because *Bmi-1*^{-/-} lymphocytes are also impaired in their proliferation³. It is also possible that other types of restricted neural progenitor (such as from other regions of the nervous system) might require *Bmi-1* for proliferation as the intrinsic properties of neural progenitors vary among regions of the nervous system⁹. Nonetheless, *Bmi-1* dependence distinguishes stem cell self-renewal from the proliferation of at least some types of restricted progenitor, providing insight that will help to elucidate these pathways further. □

Methods

Isolation of CNS and PNS progenitors

Bmi-1^{-/-} mice that had been backcrossed at least six times onto a C57BL/6 background were mated to generate E14.5 embryos, P0 pups, or 4–5-week-old adults that were genotyped by PCR (see Supplementary Information). *p16*^{-/-} mice were on an FVB background and were also genotyped by PCR. For CNS preparations, we dissected embryonic telencephalons or P0 or P30 SVZs as described^{16,27}. CNS progenitors were dissociated and plated as described in the Supplementary Information. We processed embryonic gut tissues as described⁹. Dissociated embryonic gut cells were stained with an antibody against p75 (Chemicon International), and p75⁺ cells (which include NCSCs) were sorted by a FACSVantage dual-laser flow cytometer (Becton-Dickinson) into culture plates. Guts from P0 and P30 mice were dissected in ice-cold PBS, and the outer muscle/plexus layers were peeled free from the underlying epithelium as described², dissociated and plated by pipette.

Cell culture

Cell suspensions were plated on adherent plates at clonal density, which means that cells were plated at a low density such that individual cells could form spatially distinct colonies¹⁹. This allowed us to infer the developmental and proliferative potential of single cells on the basis of the types and numbers of cells that comprised each colony. For proliferation studies, 10 μM BrdU was added to the cultures for 1 h at 37 °C before the cells were fixed and stained with an antibody against BrdU. Culture conditions are described in the Supplementary Information.

For the neurosphere formation assays (non-adherent cultures) we used ultra-low binding plates (Corning). To test the self-renewal capacity of the neurospheres, individual CNS neurospheres were selected, centrifuged at 210g for 4 min, and then mechanically dissociated and replated. PNS neurospheres were plated for 24 h into plates treated with poly-D-lysine, and then trypsin-digested, mechanically dissociated, and replated. We quantified self-renewal as the number of secondary neurospheres generated per primary neurosphere (2°NS/1°NS).

Tissue fixation and immunohistochemistry

Mice were injected intraperitoneally with 50 mg per kg (body weight) BrdU and killed after 2 h for perinatal mice and after 3 h for adult mice. The brains were fixed immediately in 4% paraformaldehyde and immunostained as described in the Supplementary Information.

The methods used for qRT-PCR, western blot analysis and microarray analysis are described in the Supplementary Information.

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Correspondence and requests for materials should be addressed to S.J.M. (seanjm@umich.edu). The microarray data entitled “Neurospheres of WT and *Bmi-1* KO” has been deposited in the GEO (Gene Expression Omnibus) database under accession number GSE611.

Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes

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Recent studies have suggested that bone marrow cells possess a broad differentiation potential, being able to form new liver cells, cardiomyocytes and neurons^{1,2}. Several groups have attributed this apparent plasticity to 'transdifferentiation'³⁻⁵. Others, however, have suggested that cell fusion could explain these results⁶⁻⁹. Using a simple method based on Cre/lox recombination to detect cell fusion events, we demonstrate that bone-marrow-derived cells (BMDCs) fuse spontaneously with neural progenitors *in vitro*. Furthermore, bone marrow transplantation demonstrates that BMDCs fuse *in vivo* with hepatocytes in liver, Purkinje neurons in the brain and cardiac muscle in the heart, resulting in the formation of multinucleated cells. No evidence of transdifferentiation without fusion was observed in these tissues. These observations provide the first *in vivo* evidence for cell fusion of BMDCs with neurons and cardiomyocytes, raising the possibility that cell fusion may contribute to the development or maintenance of these key cell types.

In order to detect cell fusion we used a method based on Cre/lox recombination, a technique extensively used to conditionally turn on or off gene expression in specific cell types or tissues, or at particular stages in development¹⁰. For this study we first used mice expressing Cre recombinase ubiquitously under the control of a hybrid cytomegalovirus (CMV) enhancer β -actin promoter¹¹ (Fig. 1a), and the conditional Cre reporter mouse line R26R¹² (Fig. 1b). In this line, the LacZ reporter gene is exclusively expressed after the excision of a loxP-flanked (floxed) stop cassette by Cre-mediated recombination (Fig. 1b). When Cre-expressing (Cre⁺) cells fuse with R26R cells, Cre recombinase excises the floxed stop cassette of the reporter gene in the R26R nuclei, resulting in expression of LacZ in the fused cells. Consequently, fused cells can be detected easily by 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) staining (Fig. 1c). This method previously failed to detect evidence of cell fusion in the pancreas¹³. For this reason, we first verified this cell fusion detection method *in vitro*.

We co-cultured bone marrow stromal cells (BMSCs) from R26R reporter mice with Cre⁺ multipotent progenitor cells isolated from postnatal brain and grown as neurospheres¹⁴. Previous studies have shown that these two cell types can fuse with embryonic stem cells *in vitro*^{6,7}. After 4 days *in vitro* (DIV), a small proportion of β -gal⁺ cells were found in these co-cultures (1 to 2 cells per 80,000 cells) (Fig. 1d). Importantly, most β -gal⁺ cells at 4 DIV had two or more nuclei, an observation that was confirmed by electron microscopy (Fig. 1e, i; see also Supplementary Fig. 1). This is consistent with the generation of β -gal⁺ cells by fusion. Notably, after 10 or 15 DIV β -gal⁺ cells formed small colonies and some of these cells were mitotic (Fig. 1g and inset). Cells in these colonies were invariably

mononucleated, suggesting that with time and cell division the nuclei of these cells fuse or supernumerary nuclei are eliminated. In addition to neurosphere cells, R26R BMSCs were also co-cultured with primary cultures of Cre⁺ fibroblasts. Three independent co-cultures did not yield positive cells, suggesting that not all cell types are equally capable of fusion in culture. To further confirm that β -gal expression was due to cell fusion, Cre⁺ neurosphere cells were labelled with 5-bromodeoxyuridine (BrdU) and then co-cultured for 5 days with R26R BMSCs. Most bi-nucleated β -gal⁺ cells in these cultures had only one of the two nuclei labelled with BrdU (Fig. 1h-k), further confirming the reliability of this method to detect fusion events.

These results confirm previous work demonstrating that cell fusion occurs spontaneously *in vitro*^{6,7}. To study cell fusion *in vivo*, R26R reporter mice were lethally irradiated, and 2 days later were grafted with bone marrow from mice constitutively expressing Cre recombinase and green fluorescent protein (GFP) under the control of the β -actin promoter (β -actin-Cre-GFP mice, see 'allogeneic bone marrow transplantation' section of Methods). We analysed the grafted mice at 2 ($n = 3$) and 4 ($n = 3$) months after transplantation. These mice showed significant levels of haematopoietic reconstitution, which was measured by flow cytometry based on the frequency of GFP⁺ cells in peripheral blood (from 54.6% to 79.8% of nucleated blood cells). Brain, liver, heart, gut, kidney, lung and skeletal muscle from these mice were serially sectioned and stained for the presence of X-gal⁺ cells. In all animals, cells labelled with β -gal were only found in brain, heart and liver, and not in the other organs studied (Table 1). As a negative control, we grafted R26R mice with bone marrow from wild-type mice. We did not find β -gal⁺ cells in these animals, demonstrating that the reporter was not inappropriately activated, even after irradiation.

BMDCs fuse with hepatocytes in fumarylacetoacetate-hydrolase-deficient mice^{8,9}. Consistent with this finding, we also observed fused hepatocytes in our grafted mice (Fig. 2). β -gal⁺ hepatocytes expressed albumin (a characteristic hepatocyte marker) (Fig. 2, h) but were negative for CD45 (a haematopoietic marker) (data not shown). Electron microscopy confirmed that these fused cells were typical hepatocytes with no features of haematopoietic cells (Fig. 2c). They contained glycogen granules, complete desmosomes and bile canaliculi (Fig. 2d). Two months after transplant most β -gal⁺ hepatocytes co-expressed the donor marker GFP (Fig. 2e, f); however, a small fraction of fused hepatocytes were GFP negative. This fraction had increased 4 months after transplantation (Table 1). This result suggests that after fusion donor genes may be inactivated/eliminated over time.

At 2 and 4 months after transplantation, β -gal⁺ cells were detected in the cerebellum, where labelled cells displayed the typical location and morphology of Purkinje cells (Fig. 3a). Two β -gal⁺ cells were embedded in plastic and serially sectioned for light and electron microscopy. Serial reconstruction of the soma of these cells demonstrated the presence of two nuclei (Fig. 3b; see also Supplementary Fig. 2). Notably, the two nuclei presented very different morphologies: one had a wrinkled surface with multiple invaginations and a single nucleolus, typical of Purkinje cells¹⁵, whereas the second nucleus showed a uniform spherical shape with multiple nucleoli, suggesting a different origin (Fig. 3b, c). Electron microscopy analysis confirmed that these cells were Purkinje neurons (Fig. 3c) with typical Purkinje cell somata and organelle distribution, including structures with the characteristics of synaptic contacts (Fig. 3d). No signs of degeneration or abnormal structures were observed in the cytoplasm of these cells (Fig. 3c, d). β -gal⁺ Purkinje cells stained positively for the Purkinje cell marker calbindin (data not shown). This is the first direct evidence, to our knowledge, showing that a neuron can fuse with a BMDC. These results suggest that previous observations of small numbers of Purkinje cells bearing markers of transplanted

bone marrow cells^{5,16,17} may have arisen by fusion rather than transdifferentiation.

The third organ where β -gal⁺ cells were found was the heart (Fig. 4). The β -gal⁺ cells were integrated into the myocardial wall and had a morphology and alignment that was indistinguishable from the surrounding cardiac muscle fibres (Fig. 4a–d). At the electron microscopy level, the fused cells had the morphology of mature cardiomyocytes, including developed filament bands and mature intercalated discs with desmosomes and GAP junctions connecting to neighbouring fibres (Fig. 4e). In addition, fused cardiomyocytes expressed cardiac troponin I (Fig. 4g). As seen in the liver, GFP was expressed in most of the fused cardiomyocytes at 2 months after transplantation (Table 1). In contrast, fused cardiomyocytes lost GFP expression at 4 months (Fig. 4h). It has been suggested that haematopoietic stem cells can partially restore the infarcted heart by transdifferentiation, giving rise to new myocardium⁴. Our results suggest that BMDs can fuse with cells within the heart to form mature cardiomyocytes, but it remains unknown

whether any new cardiomyocytes are generated as a result of this fusion.

Bone marrow cells include haematopoietic cells as well as mesenchymal cells and possibly other cell types. To test whether haematopoietic cells were participating in fusion events *in vivo* we used donor mice in which Cre recombinase was knocked into the CD45 locus (CD45-Cre). As CD45 is specifically expressed by haematopoietic cells^{18,19}, recombination should only occur in fusions involving cells of the haematopoietic lineage. To confirm the CD45-Cre expression pattern these mice were mated with R26R reporter mice. We observed widespread β -gal expression in haematopoietic stem cells, bone marrow cells, and blood cells but not in other tissues such as brain, liver, or skeletal muscle (Supplementary Fig. 3). Nonetheless, we cannot rule out the possibility of CD45-Cre expression by very rare cells in other tissues or that non-haematopoietic cells might transiently activate CD45 expression during whatever nuclear reprogramming might occur after cell fusion.

CD45-Cre bone marrow cells were injected into four lethally

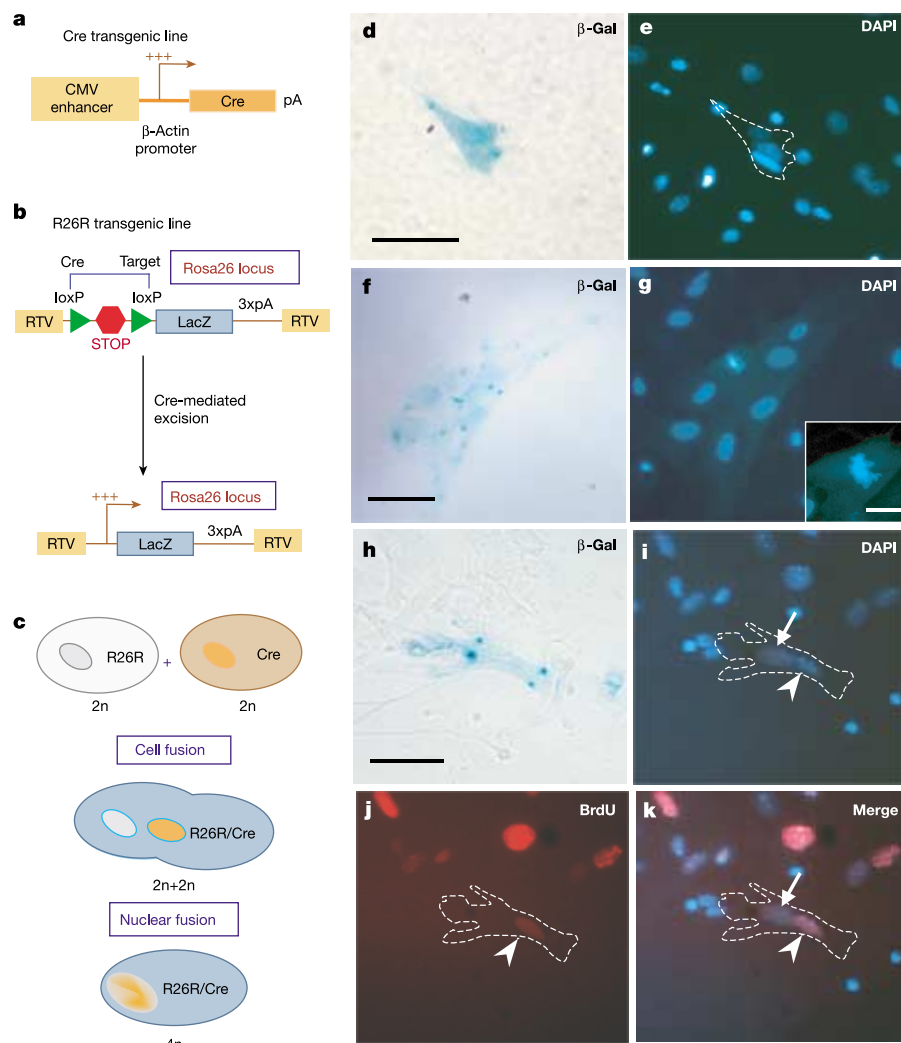


Figure 1 Method to detect cell fusion events. **a, b**, Schematic representation of the transgenes expressed by the mouse lines used. **c**, When a cell expressing Cre recombinase (**a**) fuses with a cell bearing the LacZ reporter transgene (**b**), the floxed stop cassette is excised and the LacZ reporter is expressed in the fused cell. LacZ expression can be detected by the generation of a blue precipitate after X-gal staining. RTV, Integration retroviral sequence (LTR). **d–g**, Co-cultures of R26R BMSCs with Cre⁺ neurospheres stained for β -gal and the nuclear dye DAPI (4,6-diamidino-2-phenylindole).

d, e, Multinucleated β -gal⁺ fused cell (4 DIV). **f, g**, Colony of β -gal⁺ fused cells (15 DIV). These cells were mononucleated and mitotically active, as demonstrated by a cell in metaphase (inset in **g**). **h–k**, Co-culture of R26R BMSCs with BrdU-labelled Cre⁺ neurospheres. Bi-nucleated β -gal⁺ fused cells (**h**) with one nucleus immunopositive for BrdU (**j, k**, arrowhead) and the second nucleus negative for BrdU (**i, k**, arrow). Scale bars: **d, f, h**, 20 μ m; **g** (inset), 5 μ m.

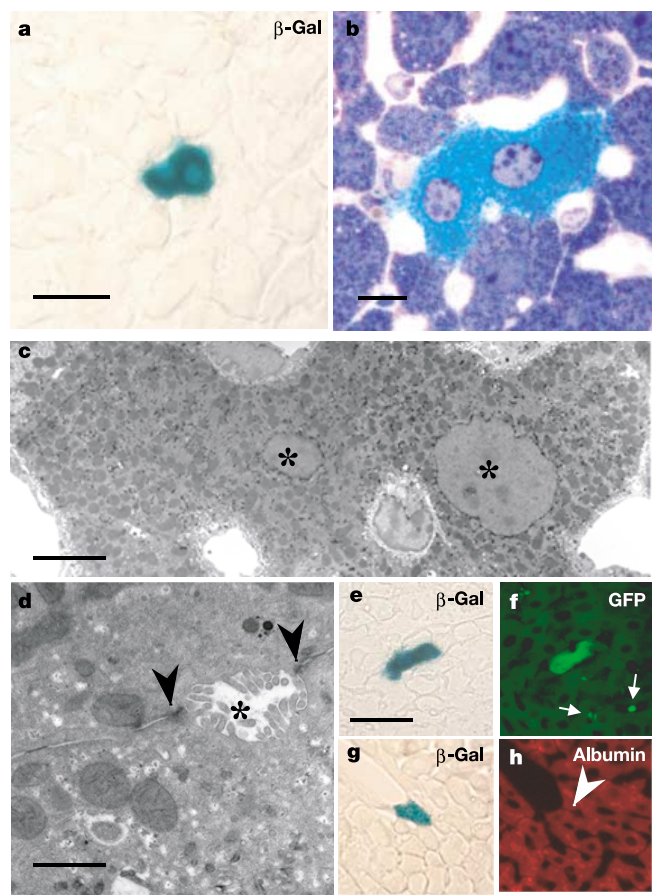


Figure 2 Fusion of hepatocytes with BMDs after bone marrow transplantation. **a**, Representative image of a β -gal⁺ hepatocyte detected in the liver of a R26R mouse transplanted with GFP⁺/Cre⁺ bone marrow. **b, c**, Semi-thin section (1.5 μ m) of a β -gal⁺ hepatocyte (light blue) counter-stained with toluidine blue (**b**), and its morphology by electron microscopy (**c**). The hepatocyte contains two nuclei (asterisks), a mitochondria-rich cytoplasm, and a very well developed endoplasmic reticulum typical of hepatocytes. **d**, High-magnification electron micrographs of a β -gal⁺ hepatocyte containing glycogen storage granules, desmosomes (arrowheads) and bile canaliculi (asterisk). **e, f**, Ten-micrometre serial section showing a β -gal⁺ hepatocyte (**e**) and the adjacent section (**f**) exhibiting GFP expression. **g, h**, Serial sections showing a β -gal⁺ hepatocyte (**g**) expressing the hepatocyte-specific marker albumin in the adjacent section (**h**). Scale bars: **a, e**, 50 μ m; **b, h**, 10 μ m; **c**, 5 μ m; **d**, 1 μ m.

irradiated R26R mice to look for fusion events (see ‘Congenic bone marrow transplantation’ section of Methods). The engrafted mice were analysed 10 months after transplantation. Consistent with the above observations, we found β -gal⁺ hepatocytes in all four mice (Table 1; see also Supplementary Fig. 3e). β -gal⁺ cardiomyocytes were found in two of the four mice, and β -gal⁺ Purkinje cells were observed in one mouse (Table 1; see also Supplementary Fig. 3). As a negative control, we lethally irradiated seven R26R mice and transplanted them with 5×10^5 R26R bone marrow cells. No β -gal⁺ cells were observed in these mice. These experiments suggest that haematopoietic cells fuse *in vivo* with cells in liver, heart and brain, but this does not rule out the possibility that other types of BMDs might also participate in fusion.

In R26R mice that had been transplanted with bone marrow cells from β -actin-Cre-GFP mice, we looked for evidence of transdifferentiation in the grafted animals. GFP⁺ cells that were negative for β -gal (that is, they did not fuse with recipient R26R cells) exhibited characteristics of microglia in the brain, of Kupffer or pit cells in the liver, and of macrophages in the heart (Supplementary Fig. 4). Each of these cell types are of haematopoietic origin^{20–22} and can fuse under certain conditions²³, making them candidates for the haematopoietic cells that fused with resident cells. In contrast, no GFP⁺/ β -gal[–] cells with the appearance of neural cells, hepatocytes, or cardiac muscle cells were observed. This suggests that cell fusion is the major mechanism by which haematopoietic cells can contribute to these tissues; however, our data do not rule out the possibility of rare transdifferentiation events, especially by other cell types or under other experimental conditions.

Our results suggest that BMDs fuse with selective cell types in three organs. We did not observe evidence of fusion in skeletal muscle, gut, kidney, or lung in these experiments. The lack of evidence for fusion in these organs could be due to a lower efficiency of Cre-mediated recombination in these tissues or lower expression of the reporter gene (Supplementary Fig. 5). Alternatively, fusion may only occur in these tissues at a lower rate or under other experimental conditions, such as after injury.

With the exception of irradiation, the mice used in these experiments were healthy and did not have any pre-existing injury or pathology. Reconstitutions involving the β -actin-Cre-GFP mice were allogeneic and therefore could have experienced graft-versus-host injury. However, reconstitutions involving CD45-Cre mice were congenic and did not involve any histoincompatibility. Although qualitatively similar results were observed in both contexts, considerable variation was observed from mouse to mouse in the extent to which fusion was observed. Therefore, the efficiency of somatic fusion *in vivo* is probably influenced by many variables.

Our results raise the fundamental question of whether fusion

Table 1 Analysis of donor-derived cells in mice receiving bone marrow transplants

Tissue and cell type (number of cells analysed)	2 months*			4 months*			10 months†
	(Transdifferentiated) β -gal [–] /GFP ⁺	(Fused) β -gal ⁺	(Fused) β -gal ⁺ /GFP ⁺	(Transdifferentiated) β -gal [–] /GFP ⁺	(Fused) β -gal ⁺	(Fused) β -gal ⁺ /GFP ⁺	
Liver/hepatocytes (20 sections, 5.5×10^5 cells per mouse)	0, 0, 0	21, 11, 9	19, 8, 7	0, 0, 0	37, 19, 7	9, 5, 2	59, 21, 12, 9
Brain (cerebellum)/Purkinje cell (60 sections, 1.5×10^6 cells per mouse)	0, 0, 0	2, 0, 0	ND	0, 0, 0	5, 3, 1	ND	5, 0, 0, 0
Heart/cardiomyocytes (25 sections, 7,750 cells per mouse)	0, 0, 0	11, 8, 9	9, 5, 6	0, 0, 0	18, 7, 2	1, 0, 0	71, 3, 0, 0
Other tissues‡	0, 0, 0	0, 0, 0	ND	0, 0, 0	0, 0, 0	ND	0, 0, 0, 0

Times indicate period after transplantation. Specimens were collected at different time points and were serially cut into 10- or 50- μ m sections. Fused cells were detected by X-gal staining. Alternating contiguous sections were screened for GFP fluorescence. $n = 3$ (2 and 4 months) or 4 (10 months); each number represents the number of cells found in the sections analysed. The number of sections analysed per mouse and the approximate total number of cells scanned in those sections are indicated in the first column. ND, not determined.

*Allogeneic transplant of whole bone marrow from β -actin-Cre-GFP mice into R26R mice.

†Congenic transplant of whole bone marrow from CD45-Cre mice into R26R mice (both on C57BL background).

‡Gut, kidney, lung and skeletal muscle.

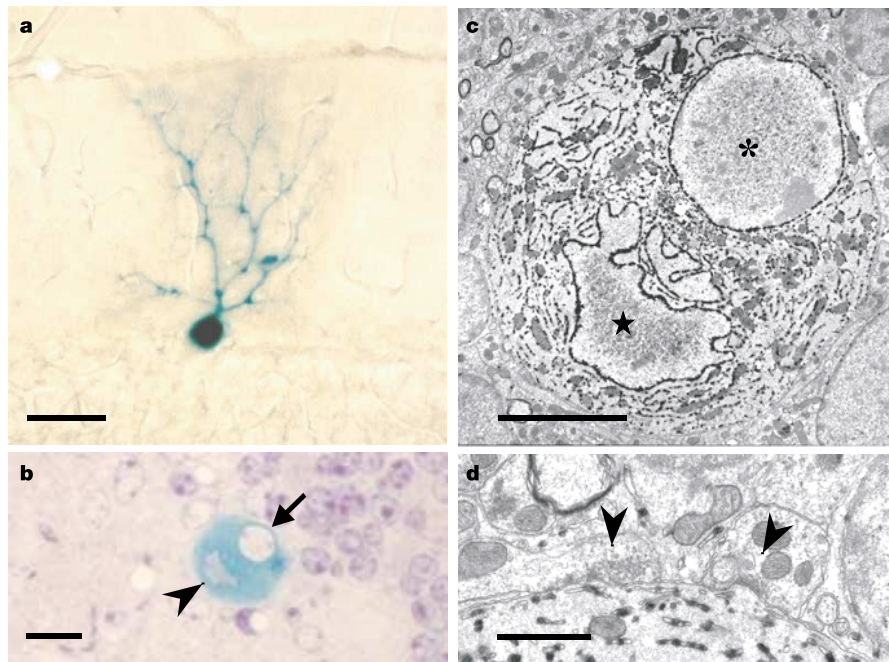


Figure 3 Purkinje cells fuse with BMDCs after bone marrow transplantation. **a**, A β -gal⁺ Purkinje neuron in the cerebellum of a R26R mouse transplanted with Cre⁺ bone marrow cells. **b**, A 1.5- μ m-thick section of the β -gal⁺ Purkinje cell soma (blue-green in colour) counter-stained with toluidine blue. This cell displays two nuclei (arrow and arrowhead) with very different morphological characteristics. **c**, Electron microscopy of the bi-nucleated fused Purkinje cell. Dark concretions in the reticulum and around the

nuclear membrane correspond to the X-gal precipitate. One nucleus is wrinkled and invaginated (star), typical of Purkinje cells, whereas the second nucleus (asterisk) has a homogeneous spherical shape, suggesting that the nuclei have different origins. **d**, Electron microscopy photomicrograph of synaptic contacts (arrowheads) in a β -gal⁺ Purkinje cell. Scale bars: **a**, 40 μ m; **b**, 10 μ m; **c**, 5 μ m; **d**, 1 μ m.

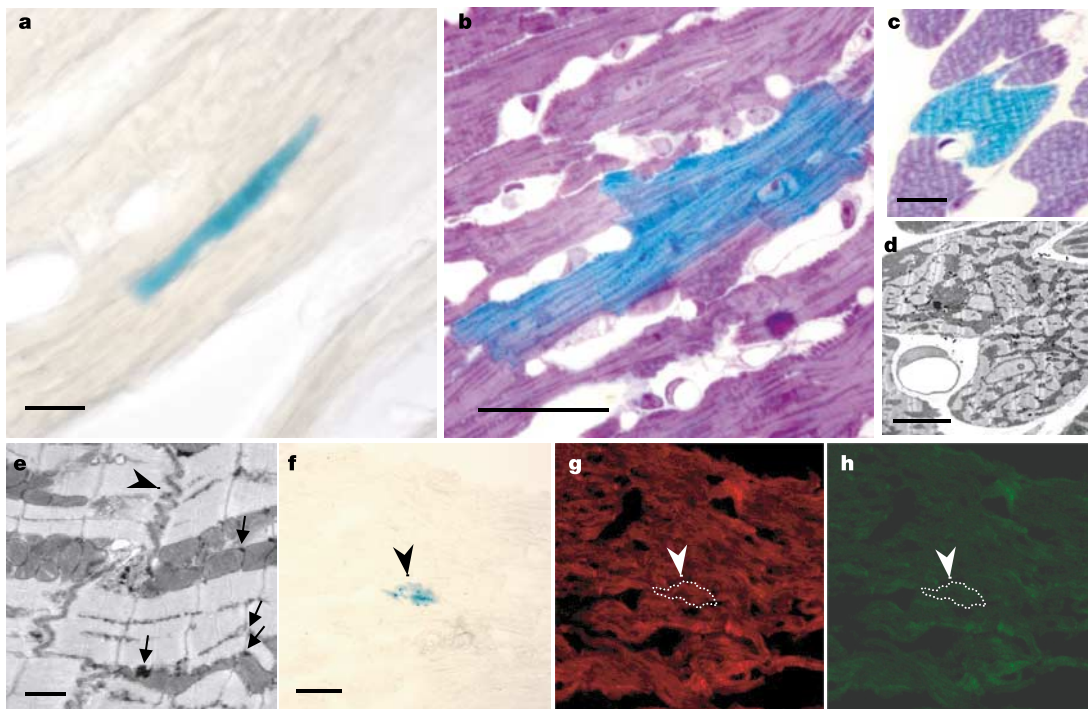


Figure 4 BMDCs fuse with cells in the heart. **a-d**, Sections at 50 μ m (**a**), 1.5 μ m (**b**, **c**) and electron microscopy (**d**) sections showing a β -gal⁺ cardiomyocyte in a R26R mouse transplanted with β -actin-Cre-GFP-expressing bone marrow cells. Note that β -gal⁺ cardiomyocytes follow the orientation of other fibres. **e**, Electron microscopy photomicrograph showing mature structures of a β -gal⁺ cardiomyocyte (β -gal

precipitates, arrows), such as intercalated discs (arrowhead) and filament bands. **f**, **g**, A group of three β -gal⁺ cardiomyocytes (**f**) were positive for cardiac troponin I (**g**) but negative for GFP (**h**). Scale bars: **a**, **f**, 25 μ m; **b**, 20 μ m; **c**, 10 μ m; **d**, 5 μ m; **e**, 1 μ m.

between haematopoietic cells and cells of the brain, liver and heart has a physiological role in the development or maintenance of these organs. Interestingly, many hepatocytes and cardiomyocytes under normal conditions have two or more nuclei^{22,24}. To our knowledge this is the first study to demonstrate Purkinje cells with two nuclei, but other studies have suggested that these neurons can be polyploid^{25,26}. Our results suggest that cell fusion may be the mechanism by which these cells become multinucleated or polyploid. Genetic material derived from blood cells may contribute through cell fusion to the survival and function of cells in different organs. Previous studies have shown that fused cells are positively selected during hepatic degeneration, helping to rescue a mutant mouse deficient for fumarylacetoacetate hydrolase^{8,9}. Our observation that fusion is a major mechanism by which BMDCs contribute to the heart, liver and brain draws into question the rationale for clinical procedures based on the idea that transdifferentiation of BMDCs can lead to the *de novo* generation of heart or brain cells. Additional studies in animal models will be required to determine whether fusion by BMDC cells can be used in reparative cell therapy. □

Methods

Cell cultures

Bone marrow cells from β -actin-Cre or R26R transgenic mice were collected by flushing tibias and femurs with RPMI medium 1640 (Gibco BRL) supplemented with 3% fetal calf serum. Red blood cells were depleted using ice-cold ammonium chloride (140 mM in Tris 17 mM), and bone marrow cells were plated at a density of $2-4 \times 10^7$ cells per 9.5 cm^2 in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL) supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 10 mg ml^{-1} glutamine (complete IMDM medium). The non-adherent cell population was removed after 48 h and the adherent BMDC layer washed once with fresh medium; cells were then continuously cultured for 1–4 weeks.

For neurospheres, brain subventricular zone from 5–10-day-old β -actin-Cre or R26R mice was collected. After papain dissociation, neurospheres were cultured and expanded in the presence of both epidermal growth factor (20 ng ml^{-1} ; Peprotech) and fibroblast growth factor-2 (10 ng ml^{-1} ; Peprotech), as described²⁷. For BrdU labelling, neurospheres were cultured for 15 min in the presence of 2 μM BrdU, washed, and expanded for two additional passages before being cultured with BMSCs. This procedure labelled 70–80% of the neurosphere cells. Fibroblasts were cultured as described previously²⁸.

Co-cultures

BMDCs and dissociated neurospheres were mixed in a 1:1 ratio and plated on Matrigel-coated dishes (BD Bioscience) at a density of 2×10^5 cells ml^{-1} in complete IMDM medium. BMDCs and primary fibroblasts were cultured in a 1:1 ratio on plastic dishes in complete IMDM medium. After 4–15 days, co-cultures were washed and fixed in 2% paraformaldehyde for 10 min and analysed by X-gal staining or immunohistochemistry. As a negative control, R26R BMDC monocultures were grown for 15 days in the presence of conditioned medium or cell extracts from Cre-expressing cells (data not shown). Cell extracts from Cre-expressing cells were freshly prepared by two freeze/thaw series and added to the culture medium.

Animal care and bone marrow transplant

Animal care and all procedures were approved by the Institutional Animal Care Committees at UCSF and the University of Michigan.

Allogeneic bone marrow transplantation

Homozygous mice expressing Cre recombinase under the control of the hybrid regulatory element CMV enhancer β -actin promoter¹¹, and homozygous mice expressing GFP under the same promoter²⁹ were bred to generate β -actin-Cre-GFP mice for use as bone marrow donors. Bone marrow cells from 8–10-week-old Cre-GFP⁺ mice were extracted as described, and $10-20 \times 10^6$ cells were intraperitoneally injected into R26R mice irradiated with a single whole-body dose of 7.5 Gy. To avoid allograft rejection, mice that received the bone marrow transplantation procedure were treated one week before transplantation and 3 weeks after transplantation with Neoral cyclosporine (100 mg l^{-1} ; Novartis) in the drinking water. Drinking water was acidified and contained neomycin sulphate (1 mg l^{-1} ; Sigma) to suppress pathogens.

Congenetic bone marrow transplantation

In an independent experiment 8–10-week-old CD45-Cre 'knock-in' mice on a C57BL/Ka-Thy1.1 background were used as donors of bone marrow cells. The generation of CD45-Cre knock-in mice will be described elsewhere (E. Schaller and K.P., manuscript in preparation). Eight-week-old R26R mice on a C57BL/Ka-Thy1.2 background were used as recipients. Approximately 5×10^5 bone marrow cells were injected into the retro-orbital venous plexus of R26R mice lethally irradiated with two doses of 5.7 Gy each. The drinking water of the transplanted mice contained neomycin sulphate (1 g l^{-1}) and polymyxin B sulphate ($1 \times 10^6 \text{ U l}^{-1}$) to suppress pathogens. On a monthly basis after transplantation, mice were bled and the peripheral blood was stained with antibodies against Thy1.1 and haematopoietic markers to confirm reconstitution.

CD45-Cre knock-in mice were bred with R26R to obtain CD45-Cre/R26R mice. The β -gal expression pattern in these mice was examined by X-gal staining of tissues and by fluorescein di- β -D-galactopyranoside (Molecular Probes) staining of haematopoietic cells. Bone marrow cells were incubated for 5 min at 37 °C with 10 mM fluorescein di- β -D-galactopyranoside in a hypotonic solution (1:1 staining medium (HBSS plus 2% calf serum):distilled water). Haematopoietic stem cells (Sca-1⁺ c-Kit⁺ Flk-2[−] lineage[−] cells)³⁰ were analysed for β -gal expression using a FACS Vantage flow-cytometer (Becton-Dickinson) (Supplementary Fig. 3a).

Tissue collection

After 2, 4 or 10 months mice were anaesthetized and transcardially perfused with 0.9% saline followed by 50 ml 4% paraformaldehyde or 2% paraformaldehyde plus 0.25% glutaraldehyde. Brain, spinal cord, liver, lung, kidney, heart, skeletal muscle and gut were dissected. Brain and one liver lobe were serially cut in 50- μm vibratome sections. The rest of the liver and other tissues were cryopreserved and frozen in optimum cutting temperature compound (Sakura-Finotec) at -80°C . Serial 10- or 50- μm sections were cut in a cryostat and stained with X-gal or by immunohistochemistry.

X-gal staining and immunohistochemistry

Specimens were placed in phosphate buffer containing 10 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and 10 mM $\text{K}_4\text{Fe}(\text{CN})_6$ along with the β -gal substrate X-gal (1 mg ml^{-1}) (Molecular Probes) at 37 °C for 8–12 h. Antibodies against albumin (A-6684; 1:100) and calbindin (C-9848; 1:1,000) were from Sigma, cardiac troponin I (sc-1881; 1:1,000) was from Santa Cruz Biotechnology, BrdU (M0744; 1:100) was from DAKO, CD45 (558750; 1:100) was from BD PharMingen, and Iba1 was a gift from Y. Imai. Secondary antibodies anti-mouse-, goat- or rabbit-IgG (H + L) (Cy-2, 1:400; Cy-3, 1:400; biotinylated, 1:500) conjugated were from Jackson ImmunoResearch.

Plastic embedding and electron microscopy

Fifty-micrometre β -gal-stained sections were post-fixed with 1% osmium and 7% glucose for 2 h, rinsed, dehydrated and embedded in araldite (Durcupan, Fluka). Semi-thin sections (1.5 μm) were cut with a diamond knife and stained lightly with 1% toluidine blue. Semi-thin sections were re-embedded in an araldite block and detached from the glass slide by repeated freezing (liquid nitrogen) and thawing. Ultra-thin (0.05 μm) sections were cut with a diamond knife, stained with lead citrate and examined under a Jeol100CX electron microscope.

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SNARE-protein-mediated disease resistance at the plant cell wall

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Failure of pathogenic fungi to breach the plant cell wall constitutes a major component of immunity of non-host plant species—species outside the pathogen host range—and accounts for a proportion of aborted infection attempts on ‘susceptible’ host plants (basal resistance)^{1–4}. Neither form of penetration resistance is understood at the molecular level. We developed a screen for penetration (*pen*) mutants of *Arabidopsis*, which are disabled in non-host penetration resistance against barley powdery mildew, *Blumeria graminis* f. sp. *hordei*, and we isolated the *PEN1* gene. We also isolated barley *ROR2* (ref. 2), which is required for basal penetration resistance against *B. g. hordei*. The genes encode functionally homologous syntaxins, demonstrating a mechanistic link between non-host resistance and basal penetration resistance in monocotyledons and dicotyledons. We show that resistance in barley requires a SNAP-25 (synaptosome-associated protein, molecular mass 25 kDa) homologue capable of forming a binary SNAP receptor (SNARE) complex with *ROR2*. Genetic control of vesicle behaviour at penetration sites, and plasma membrane location of *PEN1/ROR2*, is consistent

with a proposed involvement of SNARE-complex-mediated exocytosis and/or homotypic vesicle fusion events in resistance. Functions associated with SNARE-dependent penetration resistance are dispensable for immunity mediated by race-specific resistance (*R*) genes, highlighting fundamental differences between these two resistance forms.

Most types of plant pathogens fail to produce disease on the majority of plant species. Although ‘non-host’ resistance is the most common form of resistance, its basis is poorly understood owing to the dearth of tractable genetic systems. This contrasts with ‘race-specific’ resistance triggered by corresponding *AVIRULENCE* (*AVR*)/*R* genes in otherwise compatible host–pathogen interactions, for which many components have been identified⁵. Suicide of cells surrounding the infection site (often referred to as the hypersensitive response) typically accompanies *R*-gene-mediated resistance, and hypersensitive-response-like cell death can also be associated with non-host resistance. These drastic measures form secondary lines of defence that are normally triggered once a fungus has overcome active defences at the plant cell periphery^{3,6}.

We investigated whether the immunity of the model plant *Arabidopsis* to the barley powdery mildew *B. g. hordei* could be used to develop a system for dissecting non-host resistance. *Blumeria g. hordei* conidiospores germinated on *Arabidopsis* but most sporlings failed to enter the plant cells, accompanied by the formation of a cell wall deposition (papilla) by the plant cell directly beneath penetration attempts. About 10% of sites showed successful penetration as indicated by the presence of a fungal feeding structure (haustorium; Fig. 1a); however, most of the penetrated cells underwent hypersensitive-response-like cell death (Fig. 1b), manifested as whole-cell autofluorescence. Haustoria became encased in deposits containing callose, as revealed by aniline blue staining. Rarely, short hyphae were produced on the leaf surface (Fig. 1a), indicative of successful nutrient uptake through haustoria, before further fungal growth was invariably halted. Independent screens for *Arabidopsis* mutants allowing increased penetration by *B. g. hordei* (*pen* mutants) were performed, using either whole-cell autofluorescence or induced callose deposition as indicators of penetration. Mutants were identified for at least three genes (*PEN1*, -2 and -3; data not shown). Mutant alleles of *PEN1* were recovered from each screen.

Map-based cloning of *PEN1*, supported by the sequencing of four mutant alleles (Fig. 1c), revealed that it encodes *A. thaliana* syntaxin (At)SYP121 (ref. 7). The *pen1-1* mutation results in a stop codon early in the open reading frame and presumably leads to complete loss of *PEN1* function. *pen1-1* mutant plants allowed a sevenfold higher incidence of *B. g. hordei* penetration compared with wild-type plants, as well as a concomitant increase in the incidence of hypersensitive-response-like cell death induced by *B. g. hordei* (Fig. 1b). Further *B. g. hordei* growth was invariably arrested in *pen1-1* plants. Thus, although impairment of penetration resistance would be necessary for *Arabidopsis* to be an effective host for *B. g. hordei*, it is not sufficient. *Nicotiana tabacum* SYR1, a tobacco homologue of *PEN1/AtSYP121* (AtSYR1), has been suggested to have roles in mediating abscisic acid signalling, stomatal closing and normal growth in tobacco⁸; however, *pen1* mutants showed no discernible defects in general growth, stomatal closing ability, or root development (data not shown).

The barley–*B. g. hordei* combination also provides a useful system for the analysis of penetration resistance. Mutants of the barley *MLO* suppressor of resistance show highly effective penetration resistance against all tested *B. g. hordei* isolates. *ROR1* and *ROR2* were identified in a mutant search as genes required for full *mlo* resistance², but they also contribute to low-level basal penetration resistance expressed in ‘susceptible’ wild-type *MLO* backgrounds (Supplementary Fig. 1a). Combining mutations in *ROR1* and *ROR2* had an additive effect on susceptibility (Supplementary Fig. 1b). We isolated *ROR2* using a barley–rice syntenic-map-based cloning

approach (Supplementary Fig. 2a). A ROR2 co-segregating syntaxin gene showed a 31-amino-acid in-frame deletion in the mutant *ror2-1* line (ROR2 Δ 31) (Fig. 1c; see also Supplementary Fig. 2b). Complementation of the *ror2-1* mutation by microprojectile-mediated introduction of a genomic clone driven by the native promoter into leaf epidermal cells confirmed that the gene is ROR2 (Fig. 1d).

Transgenic *Arabidopsis* plants expressing a green fluorescent protein (GFP)–PEN1 fusion protein from the native *PEN1* promoter revealed a plasma membrane location for PEN1 (Supplementary Fig. 3a). ROR2 also showed a plasma membrane distribution in subcellular fractions analysed using a ROR2 antibody (Supplementary Fig. 3b). Of the 24 syntaxins in *Arabidopsis*⁷, PEN1 has the closest resemblance to ROR2 (62% identity and 77% similarity in the cytosolic region; other syntaxins have 55% or less identity and 75% or less similarity; Fig. 1c). A construct containing the *PEN1* coding sequence driven by the barley *ROR2* promoter complemented the penetration phenotype in *ror2-1* mutant plants (Fig. 1d). These data indicate that PEN1 and ROR2 are functionally homologous syntaxin family members possessing a specialized resistance function conserved between monocotyledons and dicotyledons. PEN1 and ROR2 also provide a mechanistic link between non-host and basal penetration resistance.

Syntaxins are members of the SNARE superfamily of proteins that mediate membrane fusion events. SNARE proteins anchored on different membranes interact through their SNARE domains to form a four-helix SNARE bundle, thereby providing much of the energy required to drive membrane fusion⁹. Plasma membrane

syntaxins (Qa-SNARE domain) typically combine with a SNAP-25 protein (Qb- and Qc-SNARE domains) and an R-SNARE protein anchored on exocytotic vesicles. Notably, the *pen1-3* substitution alters a glycine that is invariant among all nine *Arabidopsis* subgroup 1 syntaxins, at one of the 16 Qa-SNARE residues that contribute to stabilizing interactions with other SNARE proteins in membrane-fusing complexes¹⁰ (Fig. 1c).

We used a candidate gene approach to identify other factors required for *B. g. hordei* penetration resistance in barley, by silencing homologues of other SNARE proteins or SNARE-associated proteins in leaf epidermal cells. A SNAP-25 homologue was shown to be required for full resistance (construct 1, Fig. 2a), identifying it as a potential binding partner for ROR2 in a resistance-mediating SNARE complex. The product of predicted molecular mass 33.7 kDa was named HvSNAP34 (Supplementary Fig. 4). Owing to the limited silencing often obtained using this system (data not shown), the contribution of HvSNAP34 to resistance may be greater than the 4–7% penetration failure accounted for here. Cells silenced for HvSNAP34 were tested for their ability to mount resistance triggered by the *R* gene *MLA1* (ref. 11), which encodes an intracellular protein containing a nucleotide-binding domain and leucine-rich repeats (Fig. 2b). Resistance against an isolate of *B. g. hordei* containing the corresponding AVR_{MLA1} determinant was conferred specifically by *MLA1* and not by the closely related *MLA6*, indicating that HvSNAP34 is dispensable for *R*-gene-mediated resistance.

We used the cytosolic regions of wild-type ROR2 and mutant ROR2 Δ 31 proteins, as well as full-length HvSNAP34, in yeast two-

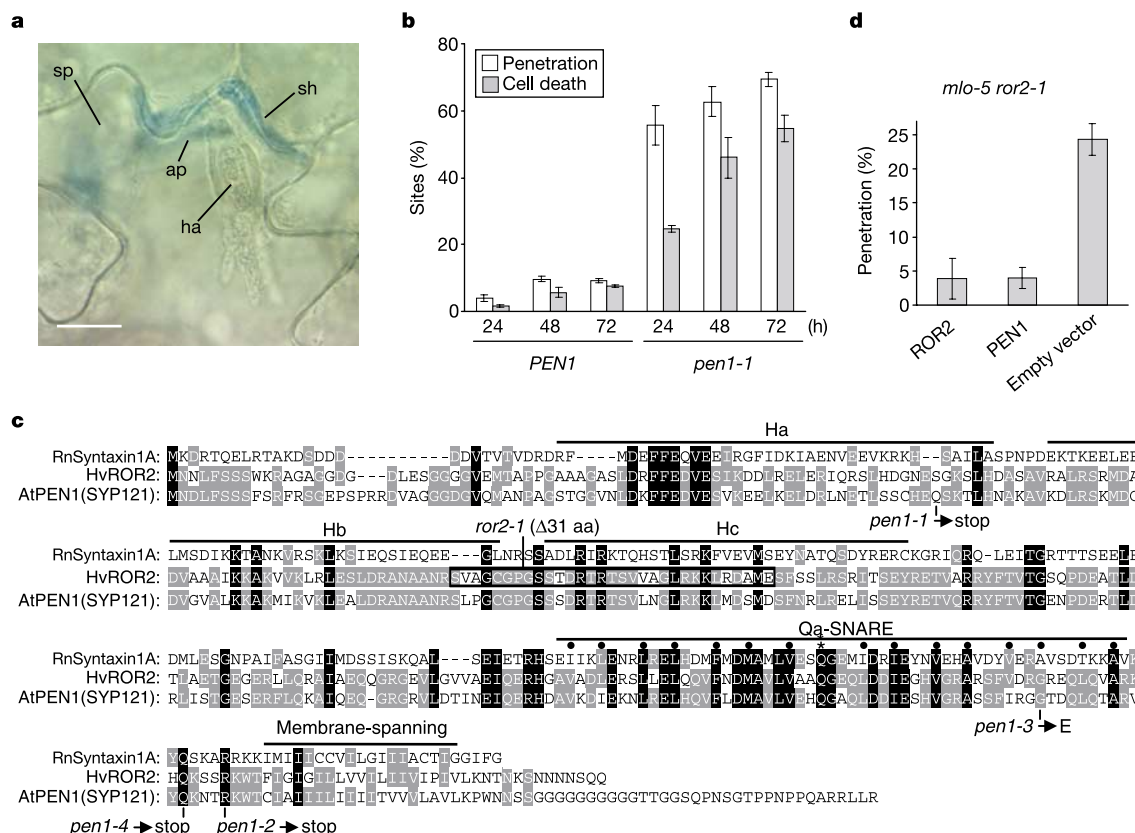


Figure 1 PEN1 and ROR2 are functionally homologous syntaxins. **a**, Multigitate haustorium (ha) formed by *B. g. hordei* in a wild-type *PEN1* *Arabidopsis* epidermal cell after successful cell wall penetration. External fungal structures are stained blue. ap, appressorium; sh, secondary hypha; sp, conidiospore. Scale bar, 10 μ m. **b**, Frequency of penetration and cell death at *B. g. hordei*–*Arabidopsis* interaction sites. The times indicated are times after inoculation. **c**, ROR2 and PEN1 mutations. Rat neuronal syntaxin

1A is included to show positions in the Qa-type SNARE domain that contribute to stabilizing ionic (asterisk) or hydrophobic (black circle) interactions with other SNARE proteins^{10,24}, and to show locations of Ha, Hb and Hc helices¹². **d**, Complementation of the *ror2-1* mutation in barley by *ROR2* and *PEN1*. Expression constructs were introduced into leaf epidermal cells of the *mlo-5 ror2-1* partially susceptible genotype.

hybrid protein interaction assays (Fig. 3a). Both forms of ROR2 interacted with HvSNAP34; however, the $\Delta 31$ deletion strongly enhanced binding to HvSNAP34 in addition to allowing formation of ROR2 $\Delta 31$ homomultimers (Fig. 3a). In other plasma membrane syntaxins, the amino terminus forms an autonomously folded bundle comprising helices Ha, Hb and Hc (Fig. 1c), which binds reversibly with the Qa-SNARE domain, suppressing interactions with other SNARE proteins and the formation of high-order homomultimers *in vitro*^{12–14}. The $\Delta 31$ deletion covers most of the predicted Hc helix (Fig. 1c). Therefore, the altered SNARE binding of ROR2 $\Delta 31$ is probably due to disruption of similar intramolecular interactions within ROR2, leading to a constitutively open state.

The ROR2 $\Delta 31$ protein produced by the endogenous *ror2-1* allele is unaltered in membrane location, and is only slightly reduced in abundance (Supplementary Fig. 3b), suggesting that its inability to confer resistance is due to disruption of a critical biochemical function requiring the region deleted in this protein. Notably, overexpressed ROR2 $\Delta 31$ acted as a potent inhibitor of resistance in a wild-type ROR2 background (Fig. 3b), probably by sequestering interacting partner(s) of ROR2 (for example, HvSNAP34) into non-functional complexes. Overexpression of ROR2 $\Delta 31$ also increased susceptibility in a mutant *ror2-1* background (Fig. 3c), suggesting that either the *ror2-1* mutant retains partial ROR2

activity, or that another syntaxin sharing interacting partner(s) with ROR2 contributes to the resistance. Cells overexpressing ROR2 $\Delta 31$ were able to mount resistance triggered by the *R* gene *MLA1* (Fig. 3d), reinforcing the notion that SNARE functions related to penetration resistance are not critical for *R*-gene-mediated resistance.

Because the requirement for SNARE proteins implies a role for membrane fusion in resistance, we examined whether the incidence of *B. g. hordei*-associated vesicles detectable by light microscopy ($>1\ \mu\text{m}$) was altered by mutations in the *MLO*, *ROR1* and *ROR2* genes. Consistent with previous observations¹⁵, large (2–3 μm) vesicles containing H_2O_2 could be observed in the host cells beneath appressoria (Fig. 4a). Vesicles appeared to aggregate and coalesce with time, and disappeared by 72 h at sites of primary penetration attempts (not shown). The appearance of vesicles was significantly influenced by mutations in each of the *MLO*, *ROR1* and *ROR2* genes, with vesicle incidence being positively associated with levels of resistance to *B. g. hordei* penetration (Fig. 4b).

Our findings obtained here (Figs 1b, 2b and 3d) and previously⁶ show that components of penetration resistance, including SNARE-related functions, are not critical for *R*-gene-mediated, race-specific resistance or for secondary lines of non-host resistance. Moreover, *PEN1*, -2 and -3 differ from genes uncovered by searches for

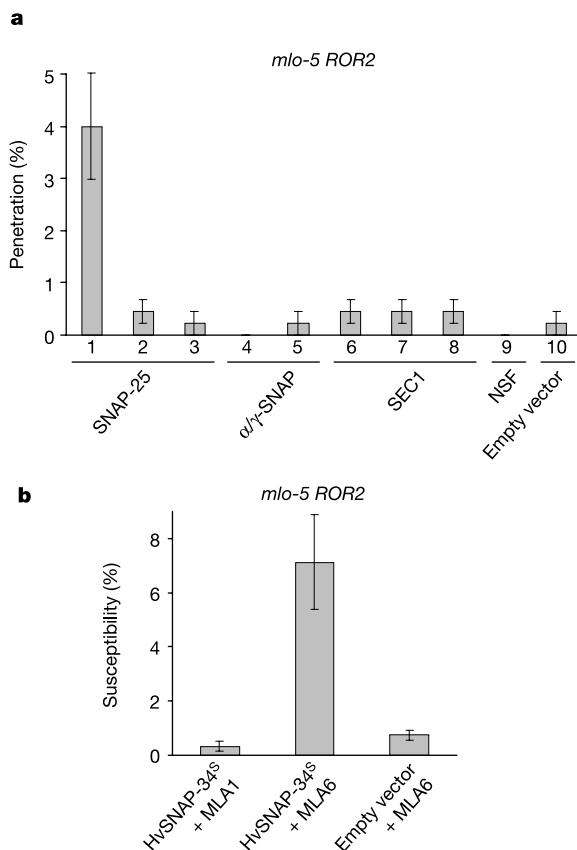


Figure 2 The barley SNAP-25 homologue HvSNAP34 is required for penetration resistance. **a**, Barley homologues of SNARE and SNARE-associated proteins were silenced in the highly resistant *mlo-5 ROR2* genotype. Representative mammalian homologues are used to indicate protein classes. Construct 1 targets HvSNAP34. GenBank accession numbers of targeted genes are listed in Methods. **b**, HvSNAP34-silenced cells retain MLA1-mediated race-specific resistance. The HvSNAP34 silencing (superscript s) construct was co-introduced with MLA1 or MLA6 *R*-gene constructs before challenge with the *B. g. hordei* isolate K1, which is recognized by MLA1 but not MLA6. Susceptibility of HvSNAP34^S plus MLA6 cells provided a control for both impairment of penetration resistance and *R*-gene-mediated resistance specificity.

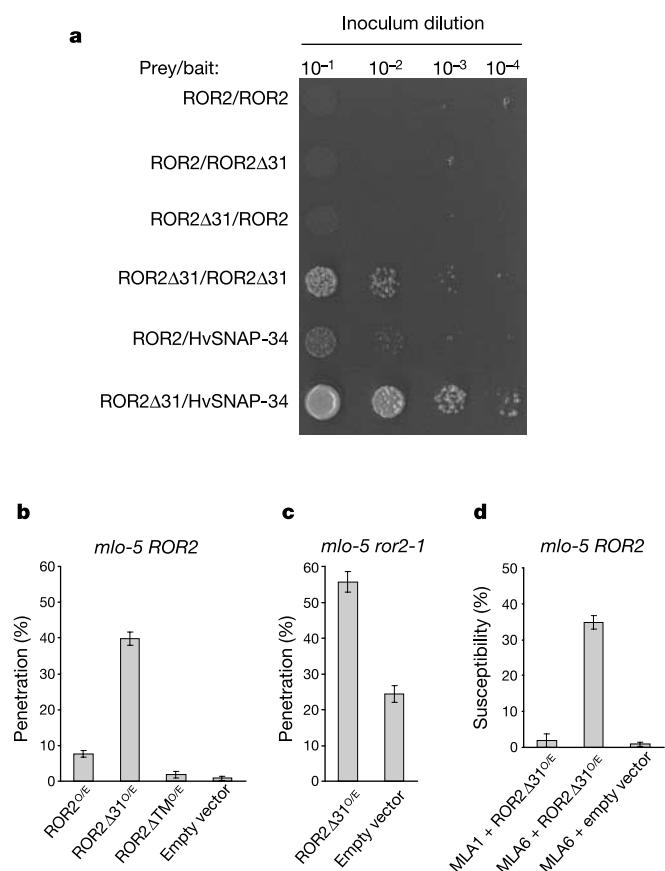


Figure 3 ROR2 interactions and overexpression. **a**, Yeast two-hybrid protein–protein interaction assays. Yeast cells were spotted on to a medium lacking histidine, upon which subsequent growth depends on interaction between bait and prey. **b**, **c**, Overexpression (O/E) constructs were introduced into barley leaves of the genotypes indicated. **d**, Cells overexpressing ROR2 $\Delta 31$ retain MLA1-mediated race-specific resistance. The overexpression construct was co-introduced with MLA1 or MLA6 *R*-gene constructs before challenge with a *B. g. hordei* strain that is recognized by MLA1 but not MLA6. Susceptibility of ROR2 $\Delta 31$ ^{O/E} plus MLA6 cells provided a control for both impairment of penetration resistance and *R*-gene-mediated resistance specificity.

enhanced disease susceptibility mutants performed in contexts of high penetration incidence in compatible host–pathogen interactions (ref. 5 and our own unpublished data). Thus, basal/non-host resistance processes responsible for halting early stages of fungal ingress seem to act independently of other resistance types.

Three lines of evidence suggest that PEN1 and ROR2 mediate resistance by participating in SNARE complexes. First, resistance also requires the Qa- and Qb-SNARE-containing protein HvSNAP34. Second, the *pen1-3* substitution alters one of the Qa-SNARE positions that contribute to stabilizing interactions with other SNARE proteins. Third, the potent resistance inhibition observed upon ROR2Δ31 overexpression, together with the enhanced binding of ROR2Δ31 to HvSNAP34, is consistent with deregulated formation of binary SNARE complexes, which normally serve as transient intermediates in assembly of ternary complexes containing the additional R-SNARE.

The plasma membrane location of PEN1 and ROR2 may facilitate exocytosis; however, the reduced incidence of *B. g. hordei*-induced vesicles in the *ror2-1* mutant defies this simple interpretation. One possibility is that, in addition to facilitating exocytosis, ROR2 may also mediate homotypic fusion of vesicles to one another, in a manner similar to KNOLLE syntaxin-dependent homotypic vesicle fusion at the growing cell plate¹⁶. Homotypic fusion could allow the vesicles to achieve a size visible by light microscopy, and might account for the fact that the vesicles are relatively large compared with most exocytotic vesicles described in animals and plants^{17,18}. One constituent of the vesicles is H₂O₂, a plant defence compound that can perform antimicrobial, cell-wall crosslinking and signalling functions¹⁹. Interestingly, the *B. g. hordei*-induced vesicles resemble, both in size and behaviour, coloured antimicrobial-compound-

containing vesicles that coalesce in sorghum leaf epidermal cells beneath sites of attempted penetration by the fungus *Colletotrichum gramminicola*²⁰. Vesicles destined for exocytosis contain R-SNAREs, which join with binary syntaxin–SNAP-25 complexes on the plasma membrane to drive membrane fusion⁹. If vesicle-anchored R-SNARE partners of ROR2 and PEN1 can be identified they may allow isolation of vesicles critical for resistance, and their cargo. □

Methods

Arabidopsis pen1 mutant screen

M₂ populations were derived by ethylmethane sulphonate treatment of *Arabidopsis* Columbia (Col-0 or Col-3 *gl1*). In the two screens yielding *pen-1*, -2 and -4, M₂ plants were inoculated with *B. g. hordei* isolate CR3, and after 48 h detached leaves were subjected to aniline blue epifluorescence staining to monitor callose²¹ deposited in response to penetration. In the screen yielding *pen1-3*, M₂ plants were inoculated with *B. g. hordei* isolate K1, and 72 h later examined with ultraviolet light (excitation filter 365/12 nm; dichroic mirror 400LP) to monitor the autofluorescence resulting from the hypersensitive-response-like cell death triggered by penetration. The *pen* mutants were deposited in the *Arabidopsis* Stock Centre.

Quantification of *pen1-1* mutant phenotype

Individual *Arabidopsis*–*B. g. hordei* interaction sites were characterized for penetration success using aniline blue and for the hypersensitive-response-like cell death using ultraviolet autofluorescence. Three repetitions, scoring 100 sites per time point and genotype, were performed.

PEN1 cloning

PEN1 was mapped using a Columbia *pen1-1* × Landsberg *erecta* F₂ population of 474 individuals using standard polymerase chain reaction (PCR)-based marker techniques.

ROR2 syntenic mapping and ROR2 sequencing

See Supplementary Information. Full-length ROR2 messenger RNA (AY246907) and genomic (AY246906) sequences were derived by rapid amplification of cloned ends and adaptor-mediated PCR methods.

Barley expression and silencing constructs

The BAC clone HvMBa693F23 was identified from the genomic DNA library of wild-type ROR2 barley cv. Morex²² by screening with a ROR2 probe. Complementation with ROR2 was performed using a HvMBa693F23 subclone containing the ROR2 open reading frame flanked by 881 base pairs (bp) of 5′ sequence and 81 bp of 3′ sequence. The PEN1 complementation construct contained the PEN1 genomic coding sequence and terminator inserted behind 3.4 kilobases of ROR2 5′ untranslated region sequence. The fusion junction followed the ATG, resulting in a D to N substitution at the third position of the encoded PEN1 protein, which is otherwise identical to PEN1.

Overexpression constructs were made using the pUBI-Adaptor-NOS vector¹¹ containing the strong constitutive maize polyubiquitin (UBI) promoter. The ROR2ΔTM construct encoding the ROR2 cytosolic region was made by introducing a T285stop mutation. We confirmed PCR-derived clones by sequencing.

Homologues of SNARE or SNARE-associated proteins were identified in tBLASTn searches of the Syngenta TMRI rice genomic sequence database (<http://portal.tmri.org/rice/RiceDescription.html>) and the Triticeae expressed sequence tag and rice genomic sequence databases (NR and HTGS) at NCBI. Silencing fragments for HvSNAP34 spanned nucleotide positions 639–982 (coding) or 1007–1275 (3′ untranslated region) of the complementary DNA (AY247208). Other genes (GenBank accession numbers AY247209 to AY247214, AJ466709 and AV833528) were targeted for silencing using fragments of 115–351 bp. The pUAMBN silencing vector contains the UBI promoter and MLA1 intron 3 located between two *attL1*–*ccdB*–*attL2* cassettes for cloning inserts in inverted orientation using Gateway technology (Life Technologies).

R gene and GUS reporter constructs have been described¹¹.

Single-cell gene expression and silencing

Gene expression and silencing in barley leaf epidermal cells was performed essentially as described¹¹. Gold microprojectiles (1.0 μm) were coated with a total of 12 μg plasmid DNA mixture per shot, using 8 μg of double-stranded RNAi construct, 0.6 μg of complementation construct but otherwise equal amounts of other constructs. Bombarded leaves were inoculated with *B. g. hordei* isolate K1 after 96 h (silencing) or 4 h (expression), and penetration frequencies were determined 48 h after inoculation. Generally, 150 interaction sites were assessed from each of three to four independent ‘shootings’ per construct combination. MLA1 and MLA6 R genes confer pre-haustorial resistance in this system due to an overexpression effect¹¹. Hence, in tests involving both penetration resistance and R-gene-mediated resistance, susceptibility was scored on the basis of haustorium formation.

Yeast two-hybrid analysis

Yeast two-hybrid tests were performed using the GAL4 system with the HIS reporter in yeast strain AH109 essentially as recommended by the suppliers (Clontech). Vectors (supplied by J. Uhrig) were made by adapting pACT2 and pAS2-1 (Clontech) to accept inserts using Gateway cloning technology (Life Technologies). PCR-derived cDNA clones were verified by sequencing, and the prey and bait constructs were co-transformed into

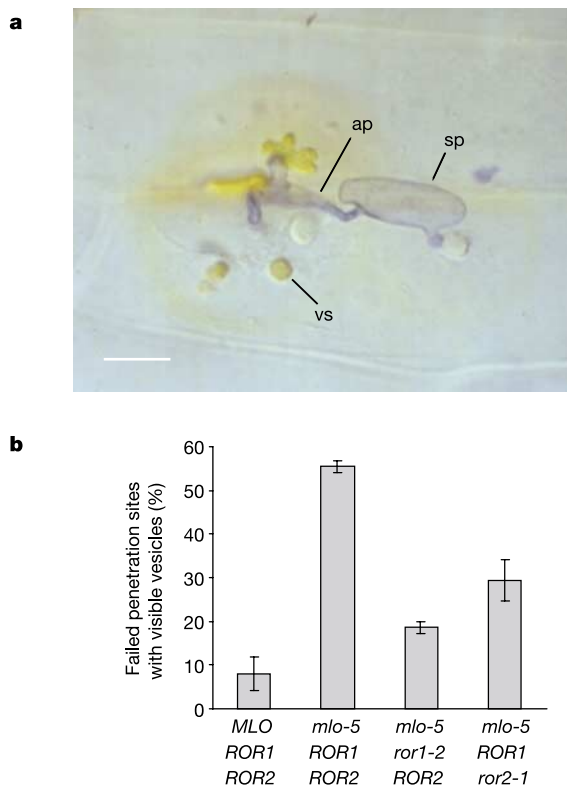


Figure 4 *Blumeria graminis hordei*-induced vesicles. **a**, A failed *B. g. hordei* penetration attempt with vesicles in barley cells. Fungal structures are stained blue. A vesicle (vs) is indicated. Brown DAB staining indicates the presence of H₂O₂. Scale bar: 10 μm. **b**, The incidence of *B. g. hordei*-induced vesicles is under genetic control. Interaction sites were classified as positive or negative for the presence of vesicles visible at ×400 magnification (approximately 1.0 μm or greater).

yeast. Liquid culture densities were equalized using absorption at 600 nm, and 10 µl of each dilution was spotted on to histidine minus medium before incubation.

Vesicle analysis

Vesicle analysis was performed on leaf segments stained with DAB to detect H₂O₂ as described¹⁵. Leaves of 7-day-old seedlings were inoculated with *B. g. hordei*, and 24 h later they were assessed by differential interference contrast microscopy for vesicles in the short cells of the adaxial epidermis²³. Per genotype, 100 sites were scored from each of three leaves. Only sites at which penetration had failed were scored.

See Supplementary Information for barley genotype analysis with *B. g. hordei*, and PEN1 and ROR2 localization.

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Bone recognition mechanism of porcine osteocalcin from crystal structure

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Osteocalcin is the most abundant noncollagenous protein in bone¹, and its concentration in serum is closely linked to bone metabolism and serves as a biological marker for the clinical assessment of bone disease². Although its precise mechanism of action is unclear, osteocalcin influences bone mineralization^{3,4}, in part through its ability to bind with high affinity to the mineral component of bone, hydroxyapatite⁵. In addition to binding to hydroxyapatite, osteocalcin functions in cell signalling and the recruitment of osteoclasts⁶ and osteoblasts⁷, which have active roles in bone resorption and deposition, respectively. Here we present the X-ray crystal structure of porcine osteocalcin at 2.0 Å resolution, which reveals a negatively charged protein surface that coordinates five calcium ions in a spatial orientation that is complementary to calcium ions in a hydroxyapatite crystal lattice. On the basis of our findings, we propose a model of osteocalcin binding to hydroxyapatite and draw parallels with other proteins that engage crystal lattices.

The primary structure of osteocalcin (OC) is highly conserved among vertebrates and contains three vitamin-K-dependent γ-carboxylated glutamic acid (Gla) residues at positions 17, 21 and 24 in porcine OC (pOC; Fig. 1a and Supplementary Fig. 1). Solution studies have shown that mature OC is largely unstructured in the absence of calcium and undergoes a transition to a folded state on the addition of physiological concentrations of calcium⁸. NMR analysis has shown that OC is a globular protein consisting of α-helical secondary structure in its folded state^{8,9}, but the detailed three-dimensional structure of OC has not been forthcoming.

To gain further insight into the structure of OC and its ability to recognize the hydroxyapatite (HA) mineral component of bone, we have determined the crystal structure of pOC at 2.0 Å using the Iterative Single Anomalous Scattering method¹⁰. Bijvoet difference Patterson map analysis detected the presence of three tightly bound Ca²⁺ ions and two S atoms corresponding to a disulphide bridge between Cys 23 and Cys 29, which together were used to phase the pOC structure. An atomic model corresponding to residues Pro 13 to Ala 49 was built into well-defined electron density (Supplementary Fig. 2) and refined to an *R*_{work} and *R*_{free} of 25.5% and 28.3%, respectively. Data collection and structure refinement statistics are summarized in Supplementary Table 1.

pOC forms a tight globular structure comprising a previously unknown fold (no matches in the DALI database¹¹) with a topology consisting, from its amino terminus, of three α-helices (denoted α1–α3) and a short extended strand (denoted Ex1; Fig. 1b). Helix α1 and helix α2 are connected by a type III turn structure from Asn 26 to Cys 29 and form a V-shaped arrangement that is stabilized

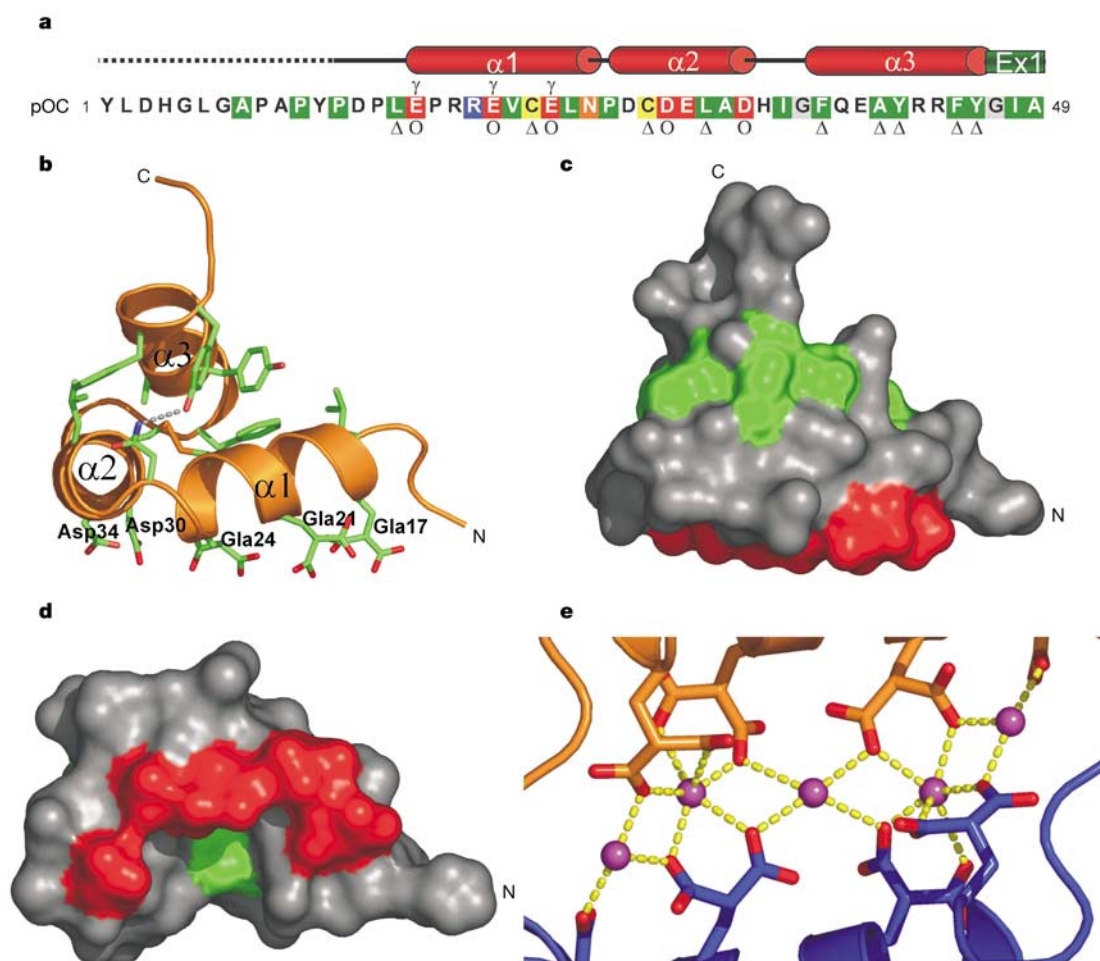


Figure 1 Structure of pOC. **a**, Protein sequence with the secondary structure elements indicated and the conserved residues highlighted (green, red, blue, yellow, orange and grey indicate conserved, acidic, basic, cysteine, asparagine and glycine residues, respectively). Positions are identified as conserved if more than 85% of the residues are identical, or similar if hydrophobic in nature (see Supplementary Information for the full sequence alignment). 'γ' indicates a Glu residue, open triangles and circles indicate hydrophobic core and Ca^{2+} -coordinating surface, respectively. **b**, Ribbon representation of the crystal structure. The N and C termini are labelled. Side chains of the Ca^{2+} -

coordinating residues and those involved in tertiary structure stabilization are shown in stick representation. Broken grey line indicates a hydrogen bond. **c**, **d**, Molecular surface representations of pOC with the surface hydrophobic patch (green) and the Ca^{2+} -coordinating surface (red) highlighted. Views in **b** and **c** are perpendicular to that in **d**. **e**, Crystallographic dimer interface. Orange and blue distinguish the two molecules. Purple spheres and the yellow broken lines represent Ca^{2+} ions and ionic bonds, respectively.

by an interhelix disulphide bridge involving Cys 23 and Cys 29. Helix $\alpha 3$ is connected to helix $\alpha 2$ by a short turn and is aligned to bisect the V-shape arrangement of helix $\alpha 1$ and helix $\alpha 2$. The three α -helices together compose a tightly packed core involving conserved hydrophobic residues Leu 16, Leu 32, Phe 38, Ala 41, Tyr 42, Phe 45 and Tyr 46. The overall tertiary structure is further stabilized by a hydrogen bond interaction between two invariant residues, Asn 26 in the helix $\alpha 1$ – $\alpha 2$ linker and Tyr 46 in helix $\alpha 3$.

Projection of conserved residues onto the molecular surface of the pOC structure (Fig. 1c, d) shows an extensive negatively charged surface centring on helix $\alpha 1$ (solvent-exposed surface area $586 \text{ \AA}^2$). Notably, all three Glu residues implicated in HA binding are located on the same surface of helix $\alpha 1$ and, together with the conserved residue Asp 30 from helix $\alpha 2$, coordinate five Ca^{2+} ions (denoted Ca1–Ca5) in an elaborate network of ionic bonds (Fig. 1e). These five Ca^{2+} ions are sandwiched between two crystallographically related pOC molecules and show both monodentate and malonate modes of chelation with extensive bridging.

In the pOC crystal structure, the Ca^{2+} ions coordinated by the Glu residues have an unexpected periodic order reminiscent of a crystalline lattice. Because Glu residues are essential for the inter-

action of OC with bone *in vivo*¹² and for the specific interaction with HA *in vitro*⁵, we investigated whether the specific atomic arrangement of bound Ca^{2+} ions in the pOC crystal structure mimics the spatial arrangement of Ca^{2+} ions in HA. To do so, we carried out a comprehensive real-space search for a spatial match between the pOC-bound Ca^{2+} ions and the Ca^{2+} ions in crystalline HA ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, space group $P6_3/m$, unit-cell dimensions $a = b = 9.432 \text{ \AA}$, $c = 6.881 \text{ \AA}$)¹³. Search solutions were ranked by root mean square (r.m.s.) deviations of distances between OC-bound and HA Ca^{2+} ions.

Unique solutions within 1 s.d. ($0.29 \text{ \AA}$) of the best solution were chosen for graphical analysis. Molecular surfaces of HA defined by the Ca^{2+} ions of our best search solutions were constructed for docking analysis (Fig. 2 and Supplementary Fig. 3). The best (r.m.s. deviation $0.44 \text{ \AA}$) and fourth best (r.m.s. deviation $0.62 \text{ \AA}$) solutions in our search correspond to the prism face (100) of HA, whereas the second (r.m.s. deviation $0.47 \text{ \AA}$) and third (r.m.s. deviation $0.61 \text{ \AA}$) best solutions correspond to the secondary prism face (110). Notably, the prism face is the predominant crystal face expressed in geological¹⁴ and synthetic HA¹⁵ and, although the predominant crystal face of HA expressed in bone has not been unambiguously

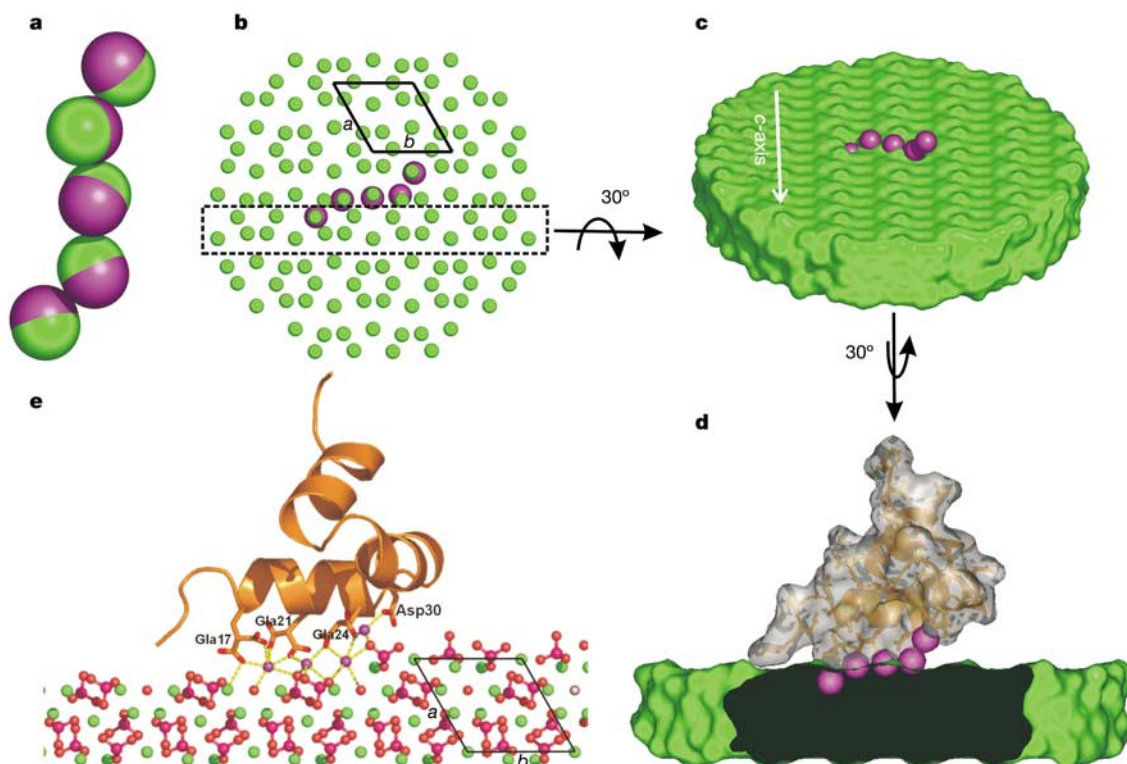


Figure 2 Model of pOC engaging an HA crystal based on a Ca^{2+} ion lattice match. Only the best search solution is shown (see Supplementary Information for a comparison of the four best solutions). **a**, Alignment of pOC-bound (purple) and HA (green) Ca^{2+} ions. **b, c**, Orientation of pOC-bound Ca^{2+} ions in a sphere of HA-Ca lattice (**b**) and on the HA surface (**c**). In **b**, the parallelogram indicates a unit cell; the box approximates the

boundary of the slab shown in **c** and **d**. **d**, Docking of pOC (orange backbone with grey semitransparent surface) on HA. **e**, Detailed view of **d** showing the Ca-O coordination network at the pOC-HA interface. Yellow broken lines denote ionic bonds. Isolated red spheres and the tetrahedral clusters of magenta and red spheres represent OH^- and PO_4^{3-} ions, respectively.

determined, atomic force microscopy¹⁶ and diffraction analysis¹⁷ indicate that the expressed face lies parallel to the crystal c axis. The best solution identified in our search also corresponds to a crystal face that lies parallel to the crystal c axis.

Although the best and second best solutions both show good lattice match statistics, only the best solution gives rise to a docking mode of pOC to HA that is free of steric clash. Using our best search solution, we generated a more detailed binding model. The coordination network of Ca-O atoms at the OC-HA interface closely mimics that in the HA crystal lattice (r.m.s. deviation Ca-O bond distance, 0.19 Å; r.m.s. deviation Ca-O-Ca bond angle, 9.63°; Fig. 2e).

The HA lattice binding mode represented by our best solution presupposes that OC engages HA with the acidic Ca^{2+} -coordinating surface as a monomer. In the crystal structure of pOC, however, the five Ca^{2+} ions are sandwiched between two crystallographically related protein molecules. If overly stable, this dimeric state could present an impediment to HA binding. To investigate whether pOC exists as a monomer or dimer in solution, we carried out sedimentation equilibrium analysis in the presence and absence of 10 mM CaCl_2 . In both cases, the sedimentation equilibrium data were best fitted to a monomer-dimer equilibrium model (Supplementary Fig. 4). The extrapolated dissociation constants (K_d) for the OC dimer were 8×10^{-4} M and 2×10^{-4} M in the absence and presence of 10 mM CaCl_2 , respectively. These K_d values are 2–3 orders of magnitude higher than the concentration of OC in human serum (0.9×10^{-6} to 7×10^{-16} M)², suggesting that OC exists as a monomer *in vivo*.

The crystal structure of pOC provides a first glimpse of the underlying interactions that may constitute biomineral recognition. The recognition of crystal lattices by proteins is important in many

biological processes, including the inhibition of ice crystal growth and the development of teeth, bone and shells. The best-characterized protein-crystal recognition system studied so far corresponds to the interaction of antifreeze proteins (AFP) with ice^{18,19}. AFPs bind to the surface of ice to modify crystal morphology and to inhibit ice growth. AFPs with different three-dimensional structures bind to different planes of ice, and the shape complementarity between the ice-binding surface of AFP and the ice crystal surface to which it binds is the primary determinant for binding specificity.

The excellent surface complementarity between the Ca^{2+} -coordinating surface of pOC and the prism face of HA suggests that pOC may also show selective binding characteristics to HA. By analogy to the antifreeze proteins, the binding of OC to HA could directly modulate HA crystal morphology and growth. In addition, when OC is bound to the surface of HA, other regions, including the carboxy terminus of the protein, which possesses chemotactic activity²⁰, would be well orientated to carry out recruitment and signal transduction functions through binding to cell surface receptors on osteoclasts⁶ and osteoblasts⁷. The structure of pOC provides a model for further functional analyses. □

Methods

Protein purification and crystallization

We purchased fresh porcine bone from the local market. Preparation of bone powder and extraction of acid-soluble proteins were done as described²¹. OC was purified to homogeneity from the acid extract by using Sep-Pak C_{18} cartridges (No. WAT043345, Waters), followed by reverse-phase HPLC using a Delta Pak C_{18} column (No. WAT011799, Waters). Protein purity was assessed by SDS-PAGE and analytical reverse-phase HPLC with a Delta Pak C_{18} column (No. 011797, Waters). Protein authenticity was determined by mass spectrometry (relative molecular mass 5,737) and amino acid composition. We obtained protein yields of 10 mg of pure OC from 30 g of bone powder.

Lyophilized OC was dissolved in 100 mM HEPES (pH 7.4) to a concentration of 10 mg ml⁻¹ (estimated by absorbance at 280 nm). We grew crystals at room temperature

by vapour diffusion from hanging drops made of equal volumes (2.5 μ l) of protein and a reservoir solution of 0.1 M HEPES, 10 mM CaCl₂ and 10% (w/v) PEG 4000, pH 7.4. Crystals of the space group P3₁21 with a unit-cell dimension of $a = 51.5$ Å, $b = 51.5$ Å and $c = 35.4$ Å grew overnight.

Crystallographic data collection and structure determination

All data were collected at beamline 17-ID of the Advanced Photon Source at Argonne National Laboratory. The X-ray wavelength was set at 1.7 Å. We cryo-cooled OC crystals in a nitrogen cold stream after transferring them to a cryo-protectant containing 30% PEG 4000, 10 mM CaCl₂ and 0.1 M HEPES, pH 7.4. Diffraction data were collected at 100 K on an Mar165 CCD detector (MarUSA) and processed with the program HKL²². Two data sets, each covering 180° rotation, were merged to increase data redundancy. Three Ca²⁺ ions and two S atoms were located and used for phase determination with the program SOLVE²³. We improved phases by solvent flattening with the program RESOLVE²⁴. The program O²⁵ was used for model building. Refinement was done with CNS²⁶ using maximum-likelihood targets. The N-terminal 12 residues are disordered and so excluded from the final model.

Modelling of OC binding to HA

To determine the HA crystal surface to which OC might bind, we wrote a Fortran program that constructs a HA lattice and carries out real-space searches. The Ca²⁺ ions at the pOC dimer interface were used as a search model as follows. First, each atom of the search model was systematically placed onto each of the two unique Ca²⁺ ions of the HA crystal lattice. Each placement then served as a pivot point on which the search model was rotated (as a rigid body to preserve the atomic arrangement) about three eulerian axes ($\theta_1, \theta_2, \theta_3$) at increments of 5° over the ranges $0^\circ < \theta_1 < 360^\circ$, $0^\circ < \theta_2 < 360^\circ$ and $0^\circ < \theta_3 < 360^\circ$. After each rotational increment, the search model was refined by least squares to the closest HA Ca²⁺ ions and the r.m.s. deviation in distance between the search model and HA Ca²⁺ ions was calculated. We sorted the unique solutions according to their r.m.s. deviation values and selected solutions within 1 s.d. of the best solution for graphical analysis. Slabs of the HA lattice corresponding to putative binding planes defined by the search solutions were constructed and pOC was docked by using the program LSQKAB²⁷ of the CCP4 (ref. 28) package.

For graphical drawing and analysis, the programs O²⁵ and Pymol²⁹ were used throughout.

Sedimentation equilibrium analysis

Sedimentation equilibrium experiments of OC in the presence and absence of Ca²⁺ at loading concentrations of 0.17, 0.23 and 0.52 mg ml⁻¹ were done at 20 °C on a Optima XL-I analytical ultracentrifuge with AN60TI rotor (Beckman). Samples were prepared by dissolving freeze-dried protein into buffer (0.1 M HEPES and 0.2 M NaCl, with or without 10 mM CaCl₂) that had been pretreated with Chelex 100. Data were acquired as an average of ten absorbance measurements at a nominal wavelength of 280 nm and a radial spacing of 0.002 cm at rotor speeds of 45,000, 50,000 and 55,000 r.p.m. Equilibrium was achieved within 16 h. We analysed data in terms of reversible monomer–dimer formation to obtain an estimate of K_d . The partial specific volume of pOC and the solvent density were calculated by the program SedNterp³⁰. Global data fits were obtained by simultaneously fitting nine sets of data to a monomer–dimer equilibrium using Origin 6.0 software (Microcal).

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ATP-dependent reduction of cysteine–sulphinic acid by *S. cerevisiae* sulphiredoxin

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Proteins contain thiol-bearing cysteine residues that are sensitive to oxidation, and this may interfere with biological function either as 'damage' or in the context of oxidant-dependent signal transduction. Cysteine thiols oxidized to sulphinic acid are generally unstable, either forming a disulphide with a nearby thiol or being further oxidized to a stable sulphinic acid^{1,2}. Cysteine–sulphinic acids and disulphides are known to be reduced by glutathione or thioredoxin in biological systems, but cysteine–sulphinic acid derivatives have been viewed as irreversible protein modifications. Here we identify a yeast protein of relative molecular mass $M_r = 13,000$, which we have named sulphiredoxin (identified by the US spelling 'sulfiredoxin', in the *Saccharomyces* Genome Database), that is conserved in higher eukaryotes and reduces cysteine–sulphinic acid in the yeast peroxiredoxin Tsa1. Peroxiredoxins are ubiquitous thiol-containing antioxidants that reduce hydroperoxides^{3–5} and con-

tol hydroperoxide-mediated signalling in mammals⁶⁻⁸. The reduction reaction catalysed by sulphiredoxin requires ATP hydrolysis and magnesium, involving a conserved active-site cysteine residue which forms a transient disulphide linkage with Tsa1. We propose that reduction of cysteine-sulphinic acids by sulphiredoxin involves activation by phosphorylation followed by a thiol-mediated reduction step. Sulphiredoxin is important for the antioxidant function of peroxiredoxins, and is likely to be involved in the repair of proteins containing cysteine-sulphinic acid modifications, and in signalling pathways involving protein oxidation.

The *Saccharomyces cerevisiae* open reading frame (ORF) YKL086W encodes a protein (Fig. 1a) of unknown function, named Srx1 for sulphiredoxin. Srx1 was initially identified by its high H₂O₂-induced expression and because the deletion of its gene causes decreased tolerance to H₂O₂ (Fig. 1b, c) and t-BOOH (not shown), which suggested its involvement in the metabolism of these oxidants. Analysis of the redox state of Srx1 in lysates of H₂O₂-treated cells showed that a significant fraction of the protein was present as an M_r = 55K disulphide-linked multimer (Fig. 2a, lanes 1, 2). To gain insight into the function of Srx1, we identified its disulphide partner(s) by affinity purification. The non-reduced purified material contained several prominent bands in the M_r range of 80, 55, 40, 35, 20 and 13K (Fig. 2b, lane 2) that resolved into two main 13 and 20K and one minor 18K bands upon reduction (lane 4). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of reduced material identified Srx1 in the 13K band, and Tsa1 and Ahp1, the two major yeast 2-Cys peroxiredoxins (Prxs)⁹⁻¹³, in the 20 and 18K bands respectively. Tsa2, another H₂O₂-inducible 2-Cys Prx closely related by structure and function to Tsa1 but much less abundant¹³, was also present in

trace quantity in the 20K band. Mass spectrometry analysis of non-reduced material identified both Srx1 and Tsa1 in the 55K band, probably in a disulphide-linked heterotrimeric form containing one Srx1 and two Tsa1 molecules, and solely Tsa1 in the 40, 35 and 20K bands, probably representing disulphide-linked dimeric and monomeric forms¹⁴. The Srx1-Tsa1 disulphide linkage was confirmed by lack of immunodection of the Srx1-containing 55K band in the H₂O₂-treated cell lysate of *Δtsa1*, a strain lacking the *TSA1* gene (Fig. 2a, lane 3). The redox association of Srx1 and Prx suggested that these proteins have linked functions.

2-Cys Prxs are obligate homodimers with two redox-active cysteines per subunit^{15,16}. They reduce hydroperoxides with the amino-terminal cysteine that oxidizes to a sulphenic acid (Cys-SOH)^{10,14,17,18}. The Cys-SOH at the catalytic site is then attacked by the carboxy-terminal cysteine of the other subunit to form a disulphide bond. In mammals, a small fraction of the enzyme is inactivated at each turnover by further oxidation of the Cys-SOH to sulphinic acid (Cys-SO₂H) instead of its condensation to a disulphide bond with the C-terminal cysteine¹⁹. Such oxidative inactivation in human 2-Cys Prx was recently shown to revert *in vivo*²⁰, but its mechanism is unknown. We analysed the *in vivo* oxidation of yeast Prxs using two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) after [³⁵S]Met pulse/chase metabolic labelling (Fig. 3a). Prx oxidation to the cysteine-sulphinic acid form, by adding an extra negative charge, shifts the 2D-PAGE mobility to a more acidic isoelectric point (pI)^{19,21}. In untreated wild-type cell extracts, Tsa1 appeared as a doublet spot, one intense spot making up about 85% of total enzyme, at the pI value position of 4.8 (±0.05), closely matching the theoretical pI of reduced Tsa1 (4.87), and the other fainter spot at a more acidic position of 4.7 (±0.05) matching the theoretical pI of Tsa1 in the sulphinic acid

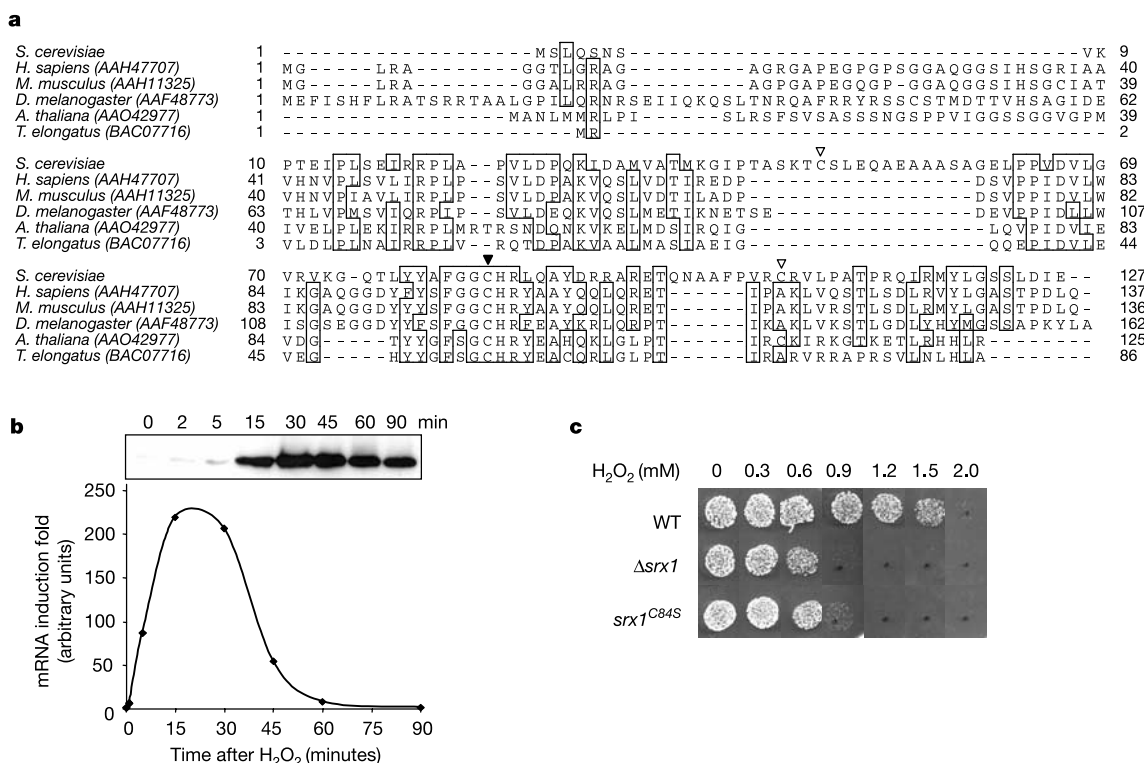


Figure 1 Srx1, a member of a new eukaryotic protein family, is induced by H₂O₂ and contributes to H₂O₂ resistance. **a**, Sequence alignment (CLUSTALW; <http://clustalw.genome.ad.jp>) of Srx1 with five representative Srx1-family members. GenBank accession numbers are indicated. Identical amino acids in at least 4/6 ORFs analysed are boxed. The conserved Srx1 active-site cysteine (black arrowhead) and the other Srx1

cysteines (white arrowhead) are indicated. **b**, Western blot (inset) and QT-RT-PCR of HA-tagged Srx1 protein and messenger RNA in H₂O₂-treated cells (400 μM) for the indicated time. **c**, Wild type (WT), *Δsrx1*, and *Δsrx1* cells carrying Srx1^{C84S} were spotted on plates containing the indicated H₂O₂ concentration.

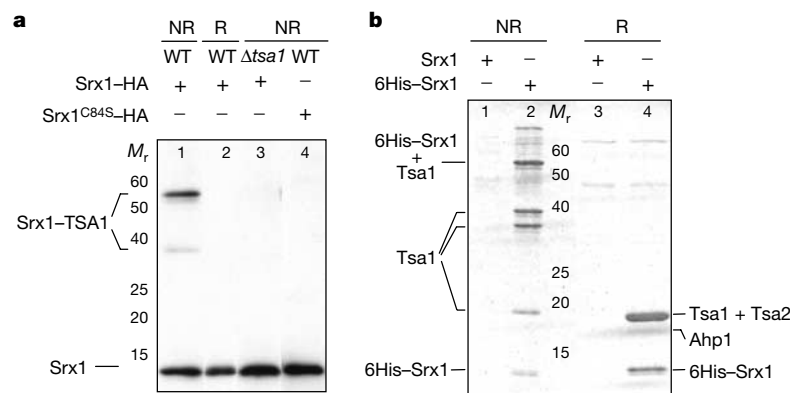


Figure 2 SrX1 is associated to Prxs non-covalently and by DTT-sensitive linkages. **a**, Western blot of HA-tagged SrX1 (lanes 1, 2, 3) or HA-tagged SrX1^{C84S} (lane 4) expressed in wild type (WT) (lanes 1, 2, 4) or $\Delta tsa1$ (lane 3) cells treated for 15 min with H₂O₂ (500 μ M), after non-reducing (NR) (lanes 1, 3, 4) or reducing (R) (lane 2) SDS-PAGE. **b**, Proteins co-purified under non-reducing conditions with 6His-tagged SrX1 (lanes 2, 4)

and the untagged SrX1 purification control (lanes 1, 3). Purified material was separated by SDS-PAGE under non-reduced (NR) (lanes 1, 2) or reduced (R) (lanes 3, 4) conditions and visualized by Coomassie blue staining. Protein bands identified by MALDI-TOF mass spectrometry are indicated.

form (4.75). Two minutes after H₂O₂ treatment (500 μ M), the proportion of oxidized Tsa1 increased at the expense of reduced Tsa1, up to about 90% of total protein, and 30 min later, the reduced/oxidized Tsa1 ratio had shifted back to that of untreated cells. Reappearance of the reduced Tsa1 spot originated from oxidized and not from *de novo* synthesized Tsa1 because protein labelling was interrupted before the analysis. To further demonstrate Tsa1 Cys-SO₂H formation and its reversion to the native Cys-SH form, we reacted dithiothreitol (DTT)-treated lysates with 4-acetamido-4'-maleimidylstilbene-2,2'-disulphonic acid (AMS) that alkylates cysteines in the free SH but not in the SO₂H state, increasing by 0.5K the protein relative molecular mass per alkylated cysteine. Cells were treated with cycloheximide (CHX) to block protein synthesis during analysis. In untreated cell extracts, reduced Tsa1 migrated as a doubly AMS-modified band (Fig. 3c). Fifteen minutes after H₂O₂ treatment, Tsa1 migrated as both doubly and singly AMS-modified species, indicating the presence of reduced and oxidized species at a 1 to 3 ratio, and 105 min later Tsa1 migrated mostly as a doubly AMS-alkylated species, demonstrating that as in human²⁰, the Cys-SO₂H form of yeast 2-Cys Prx reverts to the AMS-reactive Cys-SH state. The use of CHX delayed reversion, compared to the 2D-PAGE protocol, indicating that *de novo* protein synthesis is required for efficient Cys-SO₂H reduction.

The presence of an SrX1-Tsa1 disulphide linkage in cells treated with H₂O₂ and its transient nature, decreasing within 30 min of this treatment (not shown), suggested that the redox state of these proteins is coupled. We further tested this hypothesis by checking Tsa1 oxidation in $\Delta srx1$. In this strain, Tsa1 accumulated to its oxidized Cys-SO₂H form up to 120 min after H₂O₂ (Fig. 3b, d) and t-BOOH (not shown) treatment, indicating a role for SrX1 in Tsa1 Cys-SO₂H formation or recycling. SrX1 also affected oxidized Tsa2 but not Ahp1 (not shown), although both Prxs oxidized to the Cys-SO₂H form upon H₂O₂ treatment²² and co-purified with SrX1.

We next assayed the effect of purified SrX1 *in vitro* (Fig. 4). SrX1 supported reduction of the Cys-SO₂H form of Tsa1 present in H₂O₂-treated $\Delta srx1$ cell lysates in a dose-dependent manner when ATP was added (Fig. 4a, b). ADP could also support SrX1-dependent catalysis, but less efficiently, whereas GTP and AMP-PNP (a non-hydrolysable ATP homologue) had no effect on catalysis (not shown). Adding EDTA to the lysate inhibited SrX1-dependent Tsa1 reduction and reintroducing Mg²⁺ or Mn²⁺ (but not Fe²⁺, Ca²⁺, Cu²⁺ or Zn²⁺) restored reduction (not shown). Finally, purified SrX1 fully reduced purified and *in vitro*-oxidized Tsa1 in the presence

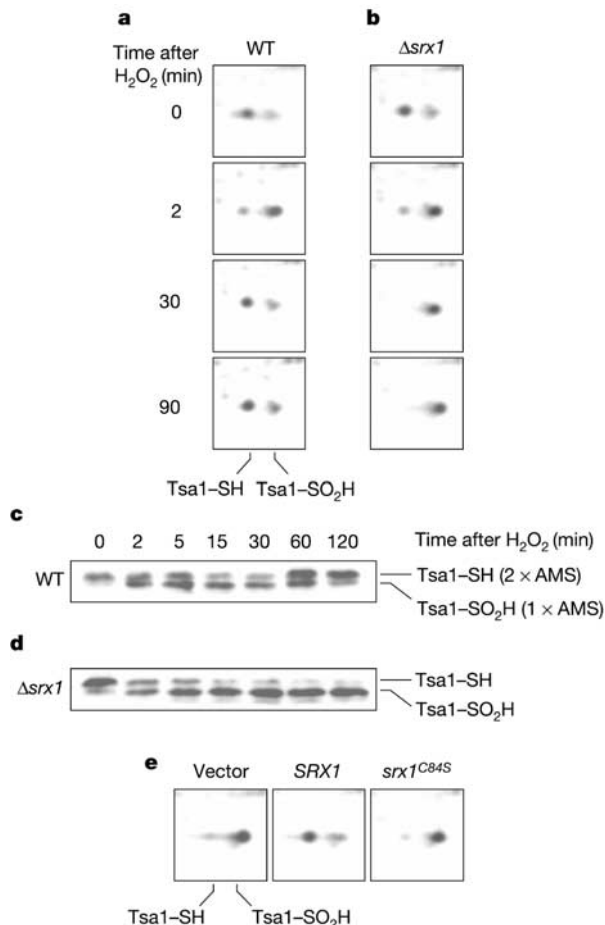


Figure 3 SrX1-dependent recycling of the Tsa1 cysteine-sulphinic acid form *in vivo*. **a**, **b**, 2D-PAGE analysis of reduced (SH) and oxidized (SO₂H) forms of [³⁵S]Met-labelled Tsa1 in WT (**a**) and $\Delta srx1$ (**b**) cells exposed to H₂O₂ (500 μ M) during the indicated period. **c**, **d**, Western blot of reduced (2 $\times$ AMS) and oxidized (1 $\times$ AMS) forms of Tsa1 from H₂O₂-treated WT (**c**) and $\Delta srx1$ (**d**) cells after *in vitro* alkylation with AMS. After inducing SrX1 expression for 15 min with H₂O₂ (100 μ M), cells were treated with cycloheximide 5 min before the H₂O₂ challenge (500 μ M). **e**, Same as in **a** and **b**, except that, as indicated, a vector empty or expressing either SrX1 or SrX1^{C84S} was reintroduced into $\Delta srx1$ cells exposed to H₂O₂ for 30 min.

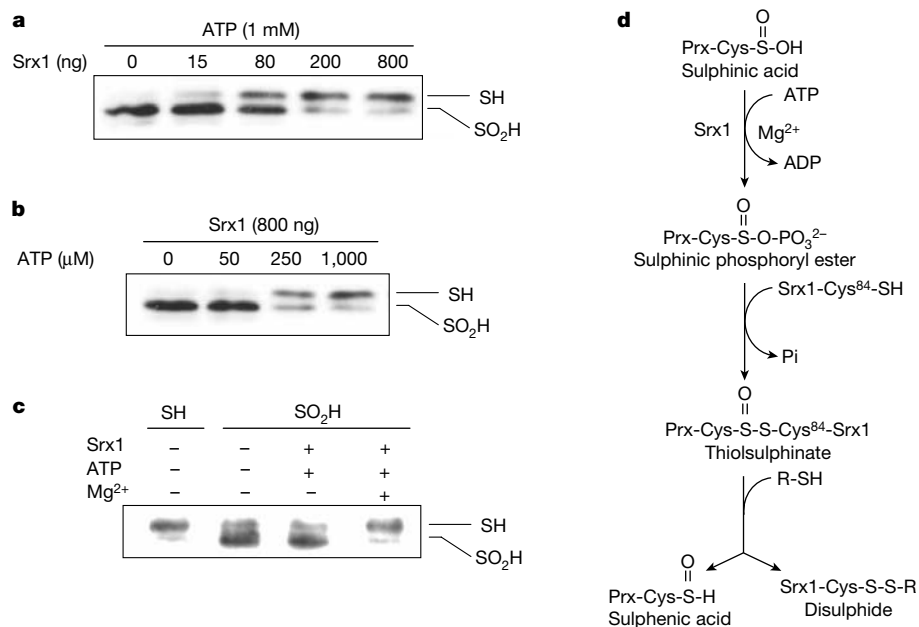


Figure 4 ATP-dependent reduction of the cysteine-sulphinic acid of Tsa1 by Srx1 *in vitro*. **a, b**, Western blot analysis of reduced (SH) and oxidized (SO₂H) forms of Myc-Tsa1 in H₂O₂-treated Δ srx1 cell lysates incubated for 15 min at 30 °C with purified Srx1 and ATP at the indicated concentrations. Reduced and oxidized Tsa1 were separated by differential

AMS alkylation as in Fig. 3. **c**, Western blot analysis of purified reduced (SH) and *in vitro* oxidized (SO₂H) forms of 6His-Tsa1 incubated for 15 min at 30 °C with purified Srx1, ATP (1 mM) and Mg²⁺ (1 mM), as indicated, and DTT (0.5 mM). **d**, Hypothetical model of the reduction of the cysteine-sulphinic acid of Tsa1 by Srx1.

of ATP, Mg²⁺ or Mn²⁺ and a dithiol reducing system, either DTT (Fig. 4c) or thioredoxin, thioredoxin reductase and NADPH (not shown); this shows that Srx1 in conjunction with a thiol-based reductant is sufficient to catalyse Cys-SO₂H reduction to the Cys-SH form. The ATP hydrolysis coupling and the specific requirement for Mg²⁺ or Mn²⁺ strongly suggested substrate phosphorylation by Srx1 as a step in the Cys-SO₂H reductive process, although such an intermediate could not be detected, probably owing to its highly unstable nature²³. The Srx1-Tsa1 disulphide linkage also suggested a thiol-based mechanism as another step in this process. We thus tested the activity of Srx1 mutants with individual substitutions of each of its three cysteines. Serine substitution of Cys 84 (Srx1^{C84S}), which is conserved in Srx1 homologues of other eukaryotes (see below and Fig. 1a), totally abolished Srx1-Tsa1 disulphide bond formation (Fig. 2a, lane 4) and reduction of the Tsa1 Cys-SO₂H form (Fig. 3e), while the other cysteine mutants had either no effect for Srx1^{C106S} or a minor effect for Srx1^{C48S} (not shown). These data indicate that the Srx1-Tsa1 linkage is contributed to by Srx1 Cys 84 and is essential for Srx1-mediated reduction of the Tsa1 Cys-SO₂H. Serine substitution of Cys 84 also abolished the *in vivo* role of Srx1 in hydroperoxide tolerance (Fig. 1c), further indicating that Srx1-dependent reduction of the Tsa1 Cys-SO₂H is important for its peroxidase function.

Cysteine-sulphinic acid in proteins cannot be reduced by typical mono- or dithiol reductants. We propose that sulphiredoxin catalyses such reduction in a multi-step process by acting both as a specific phosphotransferase and as a thioltransferase (Fig. 4d). Reduction of a cysteine-sulphinic acid probably requires its initial activation^{23,24}, which might be brought about by formation of a phosphorylated sulphinic ester, as indicated by the ATP and Mg²⁺ requirement. Such modification should enable the attack of the sulphur moiety by the Srx1 active-site cysteine, leading to the transient formation of an intermolecular thiolphosphate between Srx1 and Tsa1. Thiolphosphate is proposed to occur during oxidative stress²⁵ and is accessible to thiol-dependent reduction²⁶. Thus, once formed, the Srx1-Tsa1 thiolphosphate should be converted back to two Cys-SH by successive thiol-redox exchanges

involving initial reductive cleavage of the thiolphosphate bond to a sulphenate and a disulphide with electrons provided by DTT or thioredoxin.

BLAST (<http://www.ncbi.nlm.nih.gov>) similarity searches against the nonredundant database of the National Center for Biotechnology Information revealed that Srx1 defines a conserved protein family in lower and higher eukaryotes, all sharing the active-site cysteine (Cys 84) and a high degree of identity in the region spanning this residue (Fig. 1a), strongly indicative of conservation of the Srx1 function. In contrast, with the exception of two cyanobacteria species, no proteins similar to Srx1 were found in prokaryotes. This species difference is akin to the observation that eukaryotic Prxs are much more susceptible to oxidative inactivation than their prokaryotic counterparts, owing to an extra C-terminal α -helix and GGLG motif²⁷. Thus, Prx inactivation²⁷ and its reversion by sulphiredoxin may have been co-evolutionarily selected. We found that in addition to Tsa1, Srx1 affected the 2-Cys Prx Tsa2, both proteins having the eukaryote-shared structural features of an inactivation-sensitive enzyme, but Srx1 did not affect Ahp1, which did not retain such structural features (not shown); this is also suggestive of co-evolutionary acquisition of sulphiredoxin and Prx oxidative inactivation. As Prx inactivation might represent a regulatory feature facilitating H₂O₂ signalling in higher eukaryotes²⁷, its reversion by sulphiredoxin could add a further level of regulation on this process. The role of sulphiredoxin in Prx-mediated hydroperoxide metabolism, and its possible effects in controlling or repairing other proteins modified by cysteine-sulphinic acid formation, provides new perspectives in the biology of thiols in health and disease. □

Methods

Strains and growth conditions

The *S. cerevisiae* strains used are YPH98²⁸ (MATa, ura3-52, lys2-801^{amber}, ade2-101^{ochre} trp1- Δ 1 leu2- Δ 1) and isogenic derivatives. Δ srx1, Δ trr1 and Δ tss1 strains were made by replacing the coding region of SRX1 and TRR1 by KANMX4 and TSA1 ORF by TRP1. Cells were grown at 30 °C in YPD (1% yeast extracts, 2% bactopeptone, 2% glucose) or CASA medium (0.67% yeast nitrogen base, 0.1% casaminoacids, 2% glucose) complemented with adenine, tryptophan and uracil as required.

Plasmids

Srx1–haemagglutinin (HA), a C-terminal Srx1 fusion with two HA epitopes, and 6His–Srx1, an N-terminal fusion with a six-histidine tag, were constructed by a two-step polymerase chain reaction (PCR) amplification procedure with oligonucleotides incorporating the respective epitope sequence and then amplifying the entire ORF flanked by 400 and 200 base pairs upstream and downstream of their sequence, and cloned in *EcoRI* site of pRS316 or pRS426²⁸. Myc–Tsa1, an N-terminal Tsa1 fusion with a Myc epitope, was similarly constructed and cloned in *EcoRI*/*NorI* site of pRS316. The *SRX1* mutants carrying cysteine-to-serine substitutions were created by standard PCR-mediated site-directed mutagenesis.

Protein analyses

For 2D-PAGE, early-log-phase cell cultures ($A_{600\text{ nm}} = 0.3$) were labelled with [³⁵S]Met (100 μ Ci) for 20 min at 30 °C, chased for 3 min with cold Met (1 mM final) and Cys (0.1 mM final) and treated with H₂O₂ (500 μ M) as indicated. Cells were processed for 2D-PAGE as described²⁹. For *in vivo* Srx1–HA redox state analysis, early-log-phase cells ($A_{600\text{ nm}} = 0.3$) lysates were prepared by the trichloroacetic acid (TCA) lysis protocol³⁰. Precipitated proteins were solubilized in buffer A [Tris–Cl pH 8 (100 mM), SDS (1%), EDTA (1 mM)] containing N-ethylmaleimide (NEM) (50 mM). Extracts were separated by non-reducing or reducing 17% SDS–PAGE and Srx1–HA immunodetected with the 12CA5 monoclonal antibody. For Myc–Tsa1 cysteine derivatization by AMS, cell extracts were similarly processed by the TCA lysis protocol, except that precipitated proteins were first solubilized in buffer A containing DTT (50 mM) for 1 h at 37 °C, precipitated by TCA, and suspended in buffer A containing AMS (15 mM) for 2 h at 37 °C. Cell extracts were separated by reducing 20% SDS–PAGE and Myc–Tsa1 immunodetected with the 9E10 anti-Myc monoclonal antibody. For *in vitro* reduction, either 3 μ l of lysates (2 mg ml^{–1}) of H₂O₂-treated Δ srx1 cells containing oxidized Myc–Tsa1 or oxidized purified 6His–Tsa1 (0.5 μ g) were added in a reaction mixture buffer (RM, 80 μ l final) [Tris–Cl pH 6.8 (50 mM), KCl (100 mM)], containing baculovirus-expressed purified Srx1, ATP and MgCl₂ at the indicated concentration, and incubated for 15 min at 30 °C. 6His–Tsa1 was oxidized to the cysteine–sulphinic acid form by incubation in buffer RM containing DTT (10 mM) and H₂O₂ (1 mM) for 30 min at 30 °C, and diluted 16 times in the reaction mixture assay. To test if the thioredoxin system could substitute for DTT, the *in vitro* reduction was similarly conducted except that DTT was removed from *in vitro* oxidized Tsa1 by dialysis against buffer RM under anaerobiosis in a glove box (Jacomex) with constant nitrogen flux, and then thioredoxin (3 μ M), thioredoxin reductase (1 μ M) and NADPH (400 μ M) were added to the reaction mixture.

Purification of recombinant proteins

Srx1 was expressed in High Five insect cells using the Bac-To-Bac baculovirus expression system (Invitrogen) and purified by successive ion exchange, affinity and ion exchange high-performance liquid chromatography (8 ml Poros 50HS, 8 ml Poros 50HE, 0.8 ml Poros 20HS) (Applied Biosystems). 6His–Tsa1 was expressed in BL21 *E. coli* cells from pET28a–Tsa1 after isopropyl-thio- β -D-galactopyranoside induction according to the manufacturer's recommendations (Stratagene). Cells were suspended in lysis buffer [Tris–Cl pH 6.8 (50 mM), KCl (100 mM), DTT (2 mM), imidazole (20 mM)] supplemented with phenylmethanesulphonyl fluoride (PMSF) (1 mM), lysed by freeze–thaw cycles and sonication. Extracts were centrifuged for 30 min at 30,000g and the supernatant applied on a Ni–NTA agarose column (Qiagen). After washing the column with lysis buffer, Tsa1 was eluted with lysis buffer supplemented with imidazole (150 mM). Purity and concentration of purified proteins were determined by Coomassie blue staining after SDS–PAGE and the Bradford assay (Bio-Rad).

Purification of Srx1 partners

6His–Srx1 and Srx1 were expressed from the high copy number plasmid pRS426 in Δ trr1, a strain lacking the thioredoxin reductase gene that stabilizes disulphide bonds. Cells were grown to mid-log phase ($A_{600\text{ nm}} = 0.8$) and treated with H₂O₂ (5 mM) for 5 min, washed twice in water supplemented with NEM (10 mM), frozen, and lysed through an Eaton press in buffer C [Tris–Cl pH 8 (100 mM), NaCl (50 mM), EDTA-free complete protease inhibitors (Roche–Boehringer), PMSF (1 mM), imidazole (20 mM), NEM (10 mM)]. The cell extract was centrifuged for 90 min at 100,000g and the supernatant applied on a Ni–NTA column (Qiagen). After washing the column with buffer D [Tris–Cl pH 8 (100 mM), NaCl (50 mM)] plus imidazole (20 mM), proteins were eluted with buffer D plus imidazole (300 mM).

RNA analysis

Total RNA was extracted as described¹¹ and complementary DNAs synthesized by random hexanucleotide-primed reverse transcription (RT) from 1 μ g of total RNA. On-line quantitative PCR (Bio-Rad iCycler) was performed using the fluorescent CyberGreen method, with *SRX1* or *ACT1* specific primers, in triplicate reactions according to the supplier's recommendations. The *SRX1*/*ACT1* threshold cycle ratios were calculated using the iCycler iQ RT software (Bio-Rad).

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A monoclonal antibody to the glutamate NMDA receptor subunits 3A and 3B is available from QED. The antibody recognizes both NR3A and NR3B subunits in western blot and immunohistochemistry. NR3B is the final member of the NMDA subtype of the glutamate receptor (NMDAR) family to be cloned and characterized. When expressed in *Xenopus* oocytes, NR3A or NR3B assemble with NR1 to form excitatory glycine receptors that are unaffected by glutamate.

PatchXpress 7000A

Axon Instruments

www.axon.com

Automated workstation

PatchXpress 7000A is a high-throughput automated planar patch-clamp workstation that generates data for drug screening programmes aimed at ion-channel targets. Ion channels are implicated in pain, stroke, epilepsy, diabetes and hypertension. The PatchXpress automates the patch-clamp technique to screen drug libraries for compounds that act on ion-channel targets. It clamps 16 mammalian cells at once, then synchronizes drug delivery, electrical stimulation and data acquisition. Hundreds of compounds can be tested with minimal user intervention. The data files are managed and analysed by Axon's bioinformatics platform for electrophysiological data, DataXpress. The system can be used to produce high-content, high-quality data for both sophisticated research projects and the simpler assays typically used for high-volume drug screening.

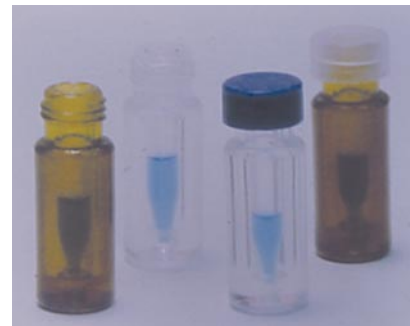
Calpain antibodies

Biodesign International

www.biodesign.com

Degenerative disease research

Biodesign International supplies calpain antibodies for researchers focusing on cell migration, differentiation, proliferation and apoptosis. Calpain, a calcium-dependent protease, has been linked with various degenerative diseases including muscular dystrophy, neurodegenerative conditions and rheumatoid arthritis. There are nine antibodies to calpain 1, 2 or 3, each having several different domain specificities. All perform in western blot applications. The products are available as purified antibodies in 150 μg vial sizes as well as custom bulk quantities.



Vial bodies: RAM containers by Finneran.

Glass/plastic vials

JG Finneran

www.jgfinneran.com

Glass fantastic

Finneran's glass/plastic vials combine a Type I low-extractable borosilicate glass insert with a thermoplastic outer vial shell. The glass is the only contact between the sample and the seal. Now available is the glass/plastic version of the popular RAM vial for robotic arm autosamplers, including those manufactured by Agilent. Available in clear or amber, these vials are used with 9-mm closures, also available from Finneran.

Biotinylated lipids

Echelon Biosciences

www.echelon-inc.com

PIPs and a sphingosine

The Echelon Biosciences product line now includes eight biotinylated phosphoinositides (PIPs) and a biotinylated sphingosine. They can be used in various assays and protein pull-down experiments. The products are available as solids or attached to streptavidin-coated plates or beads.

Sample rack

Genevac

www.genevac.co.uk

Productive drying of HPLC fractions

Genevac's new sample rack is compatible with Gilson fraction collector beds. It eliminates the need for manual handling of tubes between the fraction collector and the Genevac evaporator, improving productivity in the lab and reducing the risks of mis-identification and cross-contamination. Each block is colour-coded and laser-engraved with a unique set of tube identification numbers so blocks can be readily identified both on the fraction collector bed and following evaporation. The rack features self-locating blocks that accept standard 16×100 mm fraction collector tubes in banks of 5×5 tubes. Each rack can accommodate three

blocks, so that four racks will accept 300 tubes in 12 blocks — enough to completely fill a Genevac HT-12 evaporator for one run. The speed of evaporation of the blocks in a Genevac HT-12 is greater than can be obtained by drying the entire Gilson rack in a larger machine.



High hopes: the KNFLab Tower likes liquids.

KNFLab Tower

KNF Neuberger

www.knf.com

Three-in-one liquid handling

The new KNFLab Tower is designed to filter, pipette and aspirate a wide range of liquids quickly and efficiently. The central vacuum unit eliminates the need for separate devices for each task, but the tower is compact enough to roll (on its four castors) under the lab bench for storage. The filtration devices attach to the central unit of the tower via a hose with quick-release coupling, and pipette units with sterile filters can be fitted in the same way. Liquids can be aspirated into sealed, contamination-proof 2-litre bottles, with pressure monitored by an internal vacuum gauge.

Antibody PA1-752

Affinity BioReagents

www.bioreagents.com

Fe65 detector

RNA analyses have revealed that the Fe65 gene is predominantly expressed in the brain and in the regions most affected by pathologies associated with Alzheimer's disease. The PA1-752 antibody identifies Fe65 from purified mouse samples. It uses a western blot assay to detect a 97 K protein representing Fe65 from immunopurified mouse brain lysate. The major transcript of the Fe65 gene is a neuron-specific mRNA that encodes a

nuclear protein whose amino-terminal domain strongly activates the transcription of reporter genes. Fe65 has been implicated in a regulatory and cell-signalling mechanism because it contains two different motifs involved in protein binding — a WW domain (a variant of Src homology 3 domains) and a phosphotyrosine interaction domain (PID). PA1-752 is supplied as 100 µg of epitope affinity purified antibody.

Biotrak assay kits

Amersham Biosciences

www.amershambiosciences.com

Markers of neurodegeneration

Biotrak assay kits detect markers of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. They are enzyme-linked immunosorbent assays (ELISAs) that can detect two different forms of beta amyloid and synuclein and are validated for a variety of sample types, including brain tissue, serum, plasma, and cerebrospinal fluid. Each of the Biotrak ELISAs — β -amyloid 1-40, β -amyloid 1-42 and α -synuclein — captures and detects a specific protein in biological samples. These proteins are key biomarkers for neurodegenerative disease and may play an early and possibly contributing role in these disorders.

Primer pairs

Promega Neurosciences www.promega.com

Oligonucleotide primers for neurotrophic factor research

Promega Neurosciences offers eight sets of optimized primer pairs for analysing expression of specific mRNAs for BDNF, CNTF, GDNF, NGF, TrkB, p75/LNGFR, ChAT, and β -actin via RT-PCR. Most of the primer pairs span introns and therefore can be used to distinguish amplification of cDNA versus genomic DNA. The primers have been tested with total human and rat brain RNA in RT-PCR. They are provided in separate tubes of 100 µm each, which is sufficient for 20 RT-PCR reactions.

Preclinical assay service

Capsant Neurotechnologies

www.capsant.co.uk

A slice of life

Capsant Neurotechnologies offers assays based on organotypic hippocampal slice cultures that permit direct evaluation of potential lead molecules for drug discovery companies. The cultures closely represent brain cells and can survive outside the body. They are then developed in the lab to accurately mimic the functional behaviour of the living brain. Capsant's lead and target validation service is designed to assist with preclinical drug development and in the selection of lead candidates for clinical evaluation. Toxicity assays include

stroke models, excitotoxicity models, glutamate and hypoxia models, free radical models and amyloid toxicity models. In addition, the company offers functional assays for the determination of neuronal damage.

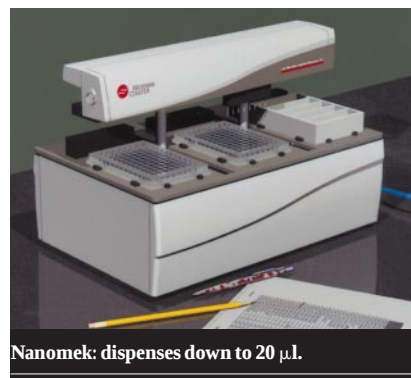
UN-SCAN-IT 6.0

Silk Scientific

www.silkscientific.com

Graph digitizing software

New UN-SCAN-IT Version 6.0 for Windows, say the makers, "just made digitizing graphs easier than ever". Version 6.0 automatically converts scanned graphs to (x,y) data using a drag and drop interface, coloured data line follower, improved grid line filters, and a graphical (x,y) data eraser. UN-SCAN-IT works with any scanner or image input device, and can be used to digitize journal graphs, strip chart output, old graphs, or any other hard-copy graph. The UN-SCAN-IT software can also integrate peak areas, smooth data, take derivatives, enhance data resolution, edit and append data, re-scale graphs, and store data in ASCII format for use in other software packages. A Macintosh version of UN-SCAN-IT, as well as a version for digitizing electrophoresis gels is also available.



Nanomek: dispenses down to 20 µl.

Nanolitre handling

Beckman Coulter

www.beckman.com

Get on down

With the ability to pipette volumes from 50 nl to 20 µl, the Nanomek extends the range of Beckman Coulter's liquid handling systems to the miniaturization of assays in genomics, proteomics and high-throughput screening applications. The Nanomek module integrates directly into the Biomek FX workstation, for automated nanolitre dispensing in 96-, 384- and 1,536-well plate formats. The new module also interfaces with Biomek FX software via an intuitive, graphical interface to facilitate method development.

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Risky business

Don't be afraid to take a chance on your career. That's the advice four panelists gave to students and postdocs at a career-fair symposium sponsored by the Harvard Biotechnology Club last week. The four panelists told the audience that they should weigh the risks at different points in their career paths.

Anthony Messina, vice-president of human development at ArQule, a chemical company in Woburn, Massachusetts, described his regrets at not taking more risks during his career. He is fixing that now by taking a management degree, which includes subjects, such as organizational change, that he used to avoid but now enjoys.

Lawyer Thomas Saunders, from Boston firm Brown Rudnick Berlack Israels, said that science students should check their motivation for going into alternative careers such as patent law. If the reason is financial security, they should think again — law school is gruelling, expensive and doesn't guarantee a stable job. Instead, graduates should go for what they really want, he said.

Mark Tepper, president of Araios, a company spun off from the University of Massachusetts, said that graduates should put themselves into potentially uncomfortable situations. Introducing yourself to chief executives and venture capitalists at biotech network meetings may be intimidating, for example, but could eventually pay dividends.

Christopher Howarth from Novartis took a risk when he left the lab to work in the human-resources office of the Swiss-based drug company. But that risk paid off — he is now in charge of global recruitment of scientific talent for the firm.

So the biggest risk of all may be not taking a chance, and finding yourself stuck in an unsatisfying career.

Paul Smaglik

Naturejobs editor



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EVENTS

Grant schemes supporting scientific entrepreneurs have induced job growth in the United States, but they haven't yet crossed the Atlantic, says Eugene Russo.

Jeffrey Alberts, a psychology professor at Indiana University in Bloomington, was trained as a scientist, not an entrepreneur. But with the help of government funding, he turned his knack for designing animal cages and other experimental apparatus into a successful small business. Alberts made the move in the 1980s after working on part of a Soviet space project that involved developmental biology experiments using rats.

The only problem was that Alberts knew little about business, so he turned to the recently established Small Business Innovation Research (SBIR) programme for help in getting his company off the ground. This US government programme offers a large number of grants and contracts through several government agencies including the defence department, the health department (especially the National Institutes of Health) and the National Science Foundation (NSF).

In 2001, after Alberts' company Star Enterprises ('rats' spelt backwards) had been in a holding pattern because of federal budget cuts, NASA awarded the firm a \$27.5-million contract for specialized animal houses for use on the International Space Station. In Alberts' case, the SBIR programme had provided key seed money for an unusual business prospect that eventually netted a major contract. "The SBIR opportunity really defined a track for me to step into," he says, referring to the business niche that the programme enabled him to develop and fill.

Although the US General Accounting Office has regularly reviewed the SBIR programme, no comprehensive evaluation of it took place until 1998, when the government asked the National Academy of Sciences to take on the task. After evaluating the defence department's SBIR programme, the academy is now reviewing the four other major participating agencies.

For scientists considering a foray into the business world, the academy's assessment of the SBIR programme's past performance gives some indication of their chances of success and the way their career may change if they set up a small business. University

deans and technology-transfer offices, meanwhile, can find out whether scientists who turn to business have been successful, and if SBIR money was wisely diverted away from directly supporting academic research. And policy-makers want to know if the programme has been cost-effective and fulfilled its role as an engine of economic growth.

The academy's assessment also provides an opportunity for European policy-makers and scientists to evaluate the SBIR programme to see whether a similar model might improve job growth and economic outlook in the European Union (EU).

The numbers show that Alberts hasn't been the only beneficiary since the SBIR's introduction. The programme now provides \$1.6 billion a year in funding and is as competitive as most federal grant programmes. In 2001, 3,136 applicants received 'phase 1' awards

to start a business, and 1,479 were awarded money for the next stage of business development.



Europe would like to ape US success in promoting small businesses, says David Audretsch.

RAPID GROWTH

Companies that get these awards grow faster than their competitors and help to provide fresh job opportunities, according to the National Academy of Sciences. This substantial federal role for small business runs counter to a rather pervasive national mythology, says Charles Wessner, director of the academy's SBIR assessment. "We think that everything starts with two guys in a garage," he says. "Sometimes it does. But usually the garage is attached to a federal laboratory, also linked to a national university and the funding comes from the NSF."

Nevertheless, it is not easy to determine whether federal support for small businesses is the best use for the money. Evaluators must consider what economists and historians call 'counterfactuals' — in other words, would a given company have been successful without government funding? Often, the answer is unclear.

In the early years of the SBIR programme, critics, especially those in academia, suggested that the money would be better spent at universities. That attitude has largely changed now, says Wessner, as a result of the



Success story: federal support allowed Jeffrey Alberts to turn his expertise at building customized animal houses into a successful company.

large number of entrepreneurial and technology-transfer success stories. It has also changed because venture capital has virtually dried up. Available venture-capital funds have fallen from a high of about \$100 billion in the 1990s to \$21 billion last year — and only about \$303 million of that is available as seed money.

Kesh Narayanan, manager of the NSF's SBIR programme, says that the foundation is "the only place in town" that can help launch early-stage companies, now that venture-capital funds have slowed. As an illustration of that fact, he points out that over the past two years the number of SBIR proposals sent to the NSF has doubled.

FOSTERING INNOVATION

In Europe, researchers and policy-makers are hungrily eyeing the entrepreneurial successes across the Atlantic and are now arguing that the EU should implement a version of the SBIR system. David Audretsch, director of the Institute for Development Strategies at Indiana University and an expert on small to medium-sized enterprises (SMEs), says that he wasn't taken seriously when he started studying SMEs in Berlin 15 years ago. He contends that Europe's reaction to the success of small businesses in the United States has gone through five stages: denial in the early 1990s, recognition followed by envy in the mid-1990s (largely due to the Silicon Valley boom), consensus in the late 1990s that a

plan of action was necessary, and, in the past few years, 'attainment' — the ongoing attempt to devise a system of small-business innovation.

Perhaps part of that attainment is a set of recommendations submitted late last year by a European Research Advisory Board (EURAB) working group. It called for, in part, an SBIR-like mechanism of funding. Although the EU's Framework Programme for Research and Technological Development currently devotes 15% of its budget to funding SMEs, that money goes towards large collaborations in which several countries might be represented.

LIMITED FRAMEWORK

Jens Rostrup-Nielsen, research director for the Haldor Topsøe chemical company in Denmark and chairman of the EURAB working group, notes that the big framework programmes are designed for research rather than innovation. The working group recommended that framework programme directors should be able to award money to investigators interested in starting companies without their having to be one of many partners or 'sub-sub-sub-contractors' to big companies. It also suggested that member states be allowed to apply the SBIR mechanism to give money directly to one company, without coming into conflict with EU regulations (a tricky prospect given the desire for free trade). Third, it recommended that SMEs within the framework programme be allowed to have one-to-one relationships with universities without being obliged to share their ideas with large industrial partners — so that, for example, an Irish company could easily collaborate with a French university.

In the European Commission's preliminary response to the report, it says that it is impossible to have an SBIR-like mechanism built into the framework programme — that there needs to be a minimum number of countries and a minimum number of partners if they are to support a project. But last spring, the commission approved a move that would give European countries the flexibility to fund potentially innovative individual companies without being beholden to cumbersome EU regulations. "This would be a step in the right direction," says Rostrup-Nielsen.

He suggests that the main difference between the United States and Europe is that in the United States a programme director can have three companies working on the same problem to create healthy competition, whereas in Europe, all three companies would have to collaborate on the same project. "It's my claim that you'll get less competition if the three have to collaborate," says Rostrup-Nielsen.

Increased competition may be a necessary ingredient of future EU efforts that seek to emulate the SBIR programme. Supporters hope that they will promote entrepreneurship, so that Alberts' US success story can be duplicated in Europe. ■

Eugene Russo is a freelance science writer in Takoma Park, Maryland.



Kesh Narayanan sees federal funding as essential now that venture capital is drying up.

Web links

Basu, P. *Bioentrepreneur* doi:10.1038/bioent766

♦ www.sba.gov/sbir